

Wanted: a new order in protein nomenclature

Biologists and others trying to make sense of the scientific literature have a hard enough time keeping up with new discoveries. Inadequate attention to nomenclature is only making the task worse.

Virtual gene cloning is here. Full-length gene sequences can be obtained in a few hours in front of a computer rather than from weeks of intensive labour at the bench. Genes, their protein products and functions, and their equivalents in other species (homologues and orthologues), are being named, identified and published at an astonishing rate.

But one side-effect is a growing chaos in nomenclature. A single protein is often studied simultaneously by a number of independent laboratories, each using their own pet name and refusing to acknowledge other names or agreeing to accept a single label. This inevitably leads to problems for anyone trying to stay abreast of the literature.

Take, for example, the EphB2 receptor, a protein involved in signalling in the brain. This tyrosine kinase was originally isolated from the chick and published in 1991 as Cek5, but was subsequently referred to as Nuk, Erk, Qek5, Tyro6, Sek3, Hek5 and Drt according to species, tissue or function. Happily in this case, following a workshop in Heidelberg, a proposal was drawn up, in consultation with the human and mouse gene-nomenclature committees, to systematically rename this protein and its family members. The scientific community at large was alerted to the agreed nomenclature of EphB2 (*Cell* **90**, 403–404; 1997), which has been used ever since.

But the speed with which genes are being identified surpasses the rate at which any consolidated naming strategy is being developed. And there are other worrying sources of confusion. In describing a gene or protein, researchers should explicitly declare all other associated names and functions. For example, a paper submitted to any

journal describing a new gene with an assigned function should unambiguously state any previous literature on an ortho/homologue to help an effective review process and to avoid unnecessary confusion of the literature, even though this might undermine the authors' ability to lay claim to a new label of their own. In practice, such referencing is sometimes not as diligent as is desirable. Thus, on occasion, what appears as the characterization of many different genes actually reflects one protein studied by many independent laboratories. What is more, two or more groups simultaneously submitting for publication papers describing the same protein in different guises are seldom able to get together and rename before publication.

How might sanity, in the form of standard nomenclature, be helped to prevail? Those administering databases could play a much more important role. A database worthy of the name should be in a position to screen new entries and to insist on conformity to a nomenclature system as a condition of registration. With such a process in place, journals could impose a requirement of prior registration as a condition of publication. Whatever the process, it remains to be seen whether rival groups would agree to refer to proteins by their first published name unless otherwise decided by all authors concerned.

For its part, *Nature* will henceforth be more rigorous in requiring that authors of papers describing the function of a protein also state all other known names of that protein the first time it is mentioned in the text. This may seem like an incremental step in dealing with the problem, but it is a start to some urgently needed improved communication within and beyond the biology community. ■

Better, cheaper, faster, more vulnerable?

NASA's policy of favouring smaller missions deserves more political support.

Officials in charge of the space science programme at NASA, the US space agency, might be forgiven for feeling put-upon this budget season. Agency head Dan Goldin has for several years been a good soldier in the rhetorical war against government spending. While other science agencies saw their budgets rise, Goldin watched his remain flat, and professed pride that NASA could do more with less. And by some measures — number of missions launched, for example — the space scientists in his organization have succeeded.

Now comes their reward — a \$184 million cut to space science proposed in the Senate, a \$265 million cut in the House of Representatives. The two chambers must reconcile their different spending bills, but the best NASA can hope for is to escape with last year's budget.

Why such harsh treatment? Congressional appropriators certainly didn't go hunting for space scientists. There is no anti-NASA lobby on Capitol Hill. But when normally friendly appropriators found themselves without enough money to satisfy noisier constituencies, space science proved a convenient account to raid.

The agency's small, cheap science missions were the hardest hit, and this may be the most sobering message to emerge from this year's

budget travail. Goldin's "better, cheaper, faster" philosophy — lots of small missions rather than a few big ones — is now firmly entrenched as NASA policy, a shift that Congress and the White House have strongly encouraged. Ironically, the strategy arose precisely because large, expensive projects were seen as more vulnerable politically. But once started, they are also hard to kill. A threat in the House to cancel the \$900 million Space Infrared Telescope Facility lasted only a few days this summer before congressional champions saw to it that the money was restored. Projects costing only \$150 million are easier to sacrifice because they have small budgets and employ small teams of scientists and engineers.

The "better, cheaper, faster" strategy is still an experiment. Its scientific and organizational merits have yet to be proven, and NASA managers have openly acknowledged that it adds risk to the already tricky business of space flight. But those controlling the space agency's budget need to let the experiment run its course. If small missions are always at risk of being scrapped or delayed, and if funding for new technologies that might reduce the cost of space missions is perpetually starved, Goldin's strategy will not have been fairly tested. ■





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Europe strengthens its hand in bioscience website talks ...

Paris

The European Molecular Biology Organization (EMBO), upset over US plans to proceed unilaterally with plans for PubMed Central, a free website for life-science papers, is to provide 500,000 euros (US\$520,000) in seed money for the creation of E-Biosci, a European counterpart to be launched in the new year.

Discussions on proposals for a global life-sciences repository, led by both the US National Institutes of Health (NIH) and EMBO, shifted sharply during the summer when Harold Varmus, director of the NIH, decided to accelerate matters and push ahead alone with the launch in January 2000 of a US site that would accept both reviewed and unreviewed manuscripts.

PubMed Central, which is estimated to cost \$1–3 million each year, already has the backing of the American Society for Cell Biology, and the US National Academy of Sciences is considering whether to take part and on what terms. But some EMBO officials are unhappy at the US decision to create PubMed Central. "We would have preferred the joint launch of a harmonized world system," says one.

EMBO's planned European operation is not intended to be a competitor to PubMed Central, as this would defeat the purpose of making the literature freely accessible from a single repository. Rather, the EMBO council felt that Europe needs to invest — and quickly — if it is to have a say in the future development of a global repository.

Most of the seed money will go to creating the computing infrastructure at the European Molecular Biology Laboratory, which runs the European Bioinformatics Institute (EBI) in Cambridge, United Kingdom.

But EMBO will seek support from the European Commission, European scientific bodies and the private sector, as its pockets are not as deep as the NIH's, and PubMed Central has a head start in its links to the popular PubMed citation and abstract database, for which Europe has no equivalent.

Varmus visited EMBO this month to discuss ways forward. The US and European systems are expected to exchange material



Key role: the European Bioinformatics Institute located at Hinxton Hall in Britain.

daily, much as happens now between genome databases at the NIH's National Center for Biotechnology Information and those at EBI — scientists accessing the system would be able to search seamlessly across one system.

The main difference between PubMed Central and E-Biosci is likely to be the content. In both systems, most of the peer-reviewed content is expected to come from existing journals, but they will differ significantly in the manner in which non-peer-reviewed material would be accepted.

PubMed Central would allow submissions from "any organization with at least three members who are principal investiga-

tors on research grants from major funding agencies", whereas EMBO favours a system in which material is accepted from anyone but with all submissions being subjected to what it calls "light peer review".

EMBO is planning for the system to be administered by a mosaic of EMBO and other scientific societies. Accepted papers will receive an EMBO stamp of approval (see *Nature* 400, 97; 1990). The principle of not accepting unreviewed preprints has gained the support of the Asia-Pacific International Molecular Biology Network (see *Nature* 400, 491; 1999).

It is still unclear whether the systems will coexist within the repository or whether common submission protocols will emerge. A determining step for both PubMed Central and E-Biosci could be the creation of an international governing body, as envisaged in the original E-Biomed proposal.

Such a body would address editorial and technical matters, as well as questions such as the terms under which commercial companies might be involved.

The United States is said to favour a governing body of eminent scientists, whereas EMBO is thought to prefer a structure comprising representatives of scientific societies, charities and organizations such as the Third World Academy of Sciences. **Declan Butler**

... and India protects its past online

New Delhi

India has launched a programme to create digital databases of its traditional knowledge — for example, of herbs with medical properties — that will be accessible to national patent offices in other countries, particularly the United States and Japan.

According to Ragunath Mashelkar, secretary to the Department of Scientific and Industrial Research, the move is intended to prevent foreigners taking out patents on such knowledge. India argued strongly for the protection of such knowledge at the recent ICSU/Unesco World Conference on Science in Budapest.

By establishing the existence of 'prior art', the databases are intended to prevent patents being granted for traditional Indian cures and remedies such as turmeric.

About two years ago, India successfully challenged the turmeric patent held by a US university (see *Nature* 389, 6; 1997). But it has failed to prevent patents on 'basmati' rice (see *Nature* 391, 728; 1998) or, more recently, on an anti-diabetic substance from bitterguard, a plant traditionally used for food and treating diabetes.

Mashelkar says that documenting traditional knowledge in India will involve translating and digitizing 250 ancient texts, ►

and examining thousands of scientific publications from the past 100 years or more. But the US\$1 million needed to create the database network is expected to save much more than that in the costs of contesting individual patents in foreign courts.

"We do not have the time or money to fight each patent," says Mashelkar. "The answer is to ensure that the patent is not awarded in the first place, by making databases of traditional knowledge available to patent examiners."

The Council of Scientific and Industrial Research has set up a unit in Pune, near Mumbai (formerly Bombay), to digitize traditional knowledge, and Mashelkar, who

heads the council, says that the work will be completed in one year.

He points out that the US Patent and Trademark Office (USPTO) suggested to India that it should create the database.

According to

USPTO, the turmeric patent might not have been issued if such a database had existed. In a letter to Mashelkar last month, USPTO official Robert Saifer admitted that the examiner who issued the patent "was not aware" of literature on the use of turmeric in wound healing in India.

K. S. Jayaraman



Hands off: India blocked a US attempt to patent turmeric.



Ion cast: Clay Armstrong, Bertil Hille and Roderick MacKinnon

Lasker awards honour three pioneers in ion channels

London

Research that pioneered the understanding of electricity in biology was this week rewarded when three scientists shared the Albert Lasker award for basic medical research. Clay Armstrong of the University of Pennsylvania School of Medicine, Bertil Hille of the University of Washington, Seattle, and Roderick MacKinnon of the Rockefeller University in New York were recognized for their work on ion-channel proteins. Lasker awards are widely seen as highly significant, as many recipients subsequently win Nobel prizes.

Ion channels are holes in the membranes of cells that control the movement of ions, governing the electrical potential of membranes and intracellular signalling. They are basic to the body's electrical system, and control phenomena such as nerve impulses, muscle contraction and cardiac rhythm.

Armstrong was recognized for his work

in elucidating the excitability of cell membranes and ion-channel gating. In the 1970s, Armstrong and Hille were among the few who believed that ion channels existed. Hille, who has worked for many years on various ion channels, is widely seen as one of the founders of this field. He showed that channels are independent physical entities within the membrane, and that they are selective to specific ions.

Potassium channels are among the most important molecules in the electrical signalling system. MacKinnon has been honoured for his work on the structure and function of these channels. He produced the first molecular description of an ion-selective channel. His 1998 paper in *Science* on the crystal structure of potassium channels was a crucial step forward for this field and sparked much new research.

Natasha Loder

Coordinate international space science, meeting told

Munich

Leading European space scientists have urged international space agencies to co-ordinate their research missions to ensure that they complement, rather than duplicate, each other.

At a meeting of the European Space Agency's (ESA) top-level science advisory committee and its science programme committee in Naples last week, scientists said that, apart from the Mars International Coordination Programme, cooperation between space-science disciplines is not as well established as it should be.

The committees, representing the astronomy, planetary-science and fundamental physics communities, met to discuss the scientific priorities for the ESA over the next decade. They noted that the major missions of the three largest space agencies are becoming increasingly similar.

ing increasingly similar.

For example, within less than a year, each will have launched X-ray observatories. The US space agency NASA's Chandra was launched in July, the ESA's XMM will launch in December (see opposite) and Japan's Astro-E will launch early next year. All three are also planning missions to Mercury.

The ESA's executive will consider ways of coordinating between disciplines, possibly at the level of the agencies' scientific advisory committees, to ensure complementarity of approach.

At the meeting, the ESA's executive said that the agency's science directorate will announce the opportunity for two medium-sized, 'flexible' missions in the coming weeks.

The executive also said that the ESA's next large, 'cornerstone' mission will be decided next June. The choice is between a journey to

Mercury, called Colombo, and an astrometry project called GAIA.

One of the two flexible missions, to be selected in a free competition next September, will probably be a contribution to the Next Generation Space Telescope, the NASA-led successor to the Hubble Space Telescope. Decisions about the second will be influenced by the choice of the next cornerstone mission, as the ESA aims for a balance between disciplines.

The science programme committee also approved the ESA's first technology mission, SMART-1, to be launched in November 2002. This will test electric-propulsion technologies for deep-space travel on a lunar mission. Small experiments will also fly on the mission, gathering X-ray and infrared spectroscopy data about the Moon's surface.

Alison Abbott

Mars bosses 'rejected trajectory revision'...

Washington

The US space agency NASA declared its Mars Climate Orbiter a total loss last week, after a targeting error apparently caused it to burn up in the Martian atmosphere just as it was about to enter orbit.

Project managers at the agency's Jet Propulsion Laboratory in Pasadena, California, were stunned on 23 September to learn that the \$125 million spacecraft, which they had predicted only a day before would skim the planet at a safe altitude of 140 km, had come only 60 km from the surface — close enough for atmospheric friction to destroy it.

The loss of a second Mars spacecraft in four attempts during the 1990s has been a distressing surprise to scientists and engineers who have come to expect pinpoint targeting of planetary spacecraft.

Investigators are likely to examine a discrepancy between two types of tracking information — one based on Doppler shifting of radio signals from the craft, the other on range data — that emerged several days before the orbiter was lost.

According to project manager Richard Cook, the discrepancy was not large, nor was it unprecedented in planetary missions. But it did prompt a discussion among project



Cook: targeting margin was thought to be adequate.

engineers, some of whom argued that the spacecraft should perform another 'trajectory correction manoeuvre' just before reaching Mars. Project managers decided against the manoeuvre, believing that the potential targeting error was within safe limits, says Cook.

The orbiter was to have spent two years studying the Martian climate, making daily weather observations similar to those returned by satellites around the Earth, says Daniel McCleese, principal investigator for one of the orbiter's two scientific instruments.

The mission would also have investigated the exchange of water between the Martian atmosphere and the surface. NASA has no plans to fly a replacement climatology mission, says McCleese, but atmospheric sensors could be included on future Mars orbiters.

Carl Pilcher, who heads NASA's planetary exploration programme, plays down the loss. He says the climate orbiter's role in

supporting another Mars mission — a lander due to touch down in December — can be filled by other means, and that some failures had to be expected when the agency was launching so many spacecraft.

But the news comes when NASA space scientists are demoralized by cuts to their budget proposed by the US Congress. The Senate last week voted for a spending bill that would trim NASA's science budget request by \$184 million next year.

This is better than the \$265 million cut recommended by the House of Representatives, but NASA says it would still result in significant losses.

Rather than cancelling missions, the agency would opt to delay programmes such as the Discovery planetary series and the MIDEX astronomy explorers. NASA estimates that some 500 research grants — a third of each year's total for academic scientists — would be in jeopardy, and the agency's advanced technology programme would be gutted.

That, in turn, would slow the development of several spacecraft missions integral to the Origins programme to investigate phenomena ranging from galactic evolution to the nature of life in the Universe. **Tony Reichhardt**

... and Europe considers insuring its X-ray satellite

London

Concerns over the possible failure of the launch of an X-ray satellite on Europe's new Ariane 5 rocket, scheduled for December, have led the European Space Agency (ESA) to consider taking out insurance on a scientific payload for the first time.

Fears for the X-ray Multi-Mirror (XMM) mission surfaced at ESA's science programme committee last week (see opposite), when the question of whether payloads should be insured was discussed.

Originally XMM was to have been the ninth launch on Ariane 5. But postponements by other customers of Arianespace, the company that administers Ariane launches, have brought XMM forward to the fourth launch of a vehicle that does not yet have an established track record.

Ariane 5's maiden flight was a disaster, exploding after lift-off and destroying ESA's Cluster mission (see *Nature* 381, 541; 1996). Arianespace has since conducted two more validation launches. The first, dogged by problems, placed its payload in the wrong orbit. The second was a success.

Ken Pounds, professor of space physics at Leicester University, says he is not aware of unusual concerns among researchers about

a launch failure. "Most people assume XMM will have the usual more than 90 per cent chance of getting into the right orbit," he says. But Pounds acknowledges that it is "not usual" for a science mission to be insured.

The insurance premium might be found from XMM's budget of 671 million euros (US\$700 million). Payload instruments supplied by national agencies are worth a further 171 million euros. Given the tensions that arise within ESA over the supply of such payloads, it may consider it worth insuring against their loss.

Meanwhile those responsible for the mission are looking closely at reports of problems with some of the detectors on the US space agency NASA's equivalent telescope, Chandra, launched in July. The front-side illuminated chip of the imaging spectrometer, ACIS, has been suffering from degradation, perhaps from the impact of



Mirror in the sky: will Ariane give it a safe lift-off?

soft protons that have penetrated the telescope's shield.

Martin Weisskopf, project scientist for Chandra, says NASA has been working closely with ESA and with researchers who may be affected by the problem. So far, the only effect appears to be a reduction in the efficiency of the telescope. "Our mission is not impaired," he says. "It is not a crisis, [although] it is annoying and a nuisance."

But Pounds says his group is studying Chandra's performance "keenly". He adds: "We need to satisfy ourselves that there is no parallel danger for XMM, and ensure we are not vulnerable to similar problems."

NASA is still working on the source of the problem. One theory is that the damage results partly from Chandra's deep-space orbit. This brings it into contact with the Earth's outer radiation belts, in particular the Van Allen belt, which consists of high-energy electrons, protons and other energetic particles.

ACIS may have been damaged by particles passing down the telescope while in this belt. NASA has removed the instruments from the focal plane of the telescope during this period. Weisskopf says the degradation has ceased.

Natasha Loder & Alison Abbott

Monbusho pleads for watered-down agency bill for universities

Tokyo

Japan's education ministry last week approved the government's plan to turn national universities into 'agencies' with greater administrative independence, provided that they are allowed to continue to give priority to education and research.

The move would add Japan's 99 national universities to a list of government-run institutions, including museums and national research institutes, that are to be transformed into semiautonomous agencies. The plans are part of the government's bid to increase administrative efficiency by placing the institutions under the control of a separate management system (see *Nature* 389, 897; 1997).

Rather than complying with the government's agency bill, which sets targets based on its rationalization plan, the Ministry of Education, Science, Sports and Culture (Monbusho) is asking for a separate bill for universities to maintain the standard of research and education.

According to the proposed bill, performance-related targets would be reviewed every five years, instead of on the shorter, three- to five-year timescale suggested in the agency bill, and the evaluation be carried out by an independent assessment body that Monbusho plans to set up next April (see *Nature* 400, 704; 1999).

The proposed bill also promises that some policy decisions, such as the selection of performance-related targets and the appointment of university deans, would reflect the universities' opinions.

Monbusho also insists that university staff should retain their civil-service status, despite pressure from the government to reduce the number of civil servants. The government hoped to reduce Japan's civil servants by 25 per cent in ten years by giving agency status to national universities, which have more than 125,000 staff.

Akito Arima, the education minister, said last week at a meeting of deans of national universities that university reform should be separated from the government's aim to cut central-government employees. "The main aim of the reform is to give the universities more freedom and autonomy, and to enhance their level of education and research," said Arima.

But Monbusho's proposed bill has been criticized by members of the ruling Liberal Democratic Party and government ministries as being too easy on universities. Many expect it to face strong opposition before its final approval next April. **A.S.**



Dambuster: the Shihkang reservoir was damaged by the earthquake that struck Taiwan last week.

Taiwan's high-tech industries shaken not stirred by quake

Tokyo

Last week's earthquake in Taiwan, which killed at least 2,000 people and injured a further 8,000, is likely to damage the nation's high-tech industry, especially its semiconductor companies, which account for seven per cent of the world market.

But officials at the Hsinchu Science Park, where activities were brought to a standstill, predict that the impact will only be short term.

The science park, located in northeastern Taiwan, is home to almost 300 companies and research centres, of which 50 are from overseas, involved in manufacturing integrated circuits and computers, optoelectronics, telecommunications, biotechnology and materials.

Key semiconductor companies reported no serious damage to their facilities. But power cuts and aftershocks are expected to delay the recovery of their operation for another month. Taiwan Electric say the power supply to industrial and business areas is unlikely to return to normal until next week.

Industry analysts are predicting a loss of at least NT\$10 billion (US\$315 million) for companies based at the science park and a total loss of NT\$100 billion for Taiwanese industry as a whole. But Steve Hsieh, vice-chairman of Taiwan's National Science Council, says that almost half of the park's losses can be recovered this year as the earthquake actually caused very little damage.

In Japan, where many electronics companies import semiconductors and liquid-crystal devices from Taiwan, there is concern that the earthquake could lead to a serious shortage of computer parts — the price of semiconductors in Japan increased by 20 per cent immediately after the earthquake.

"We have to rethink our strategy, as delayed production [of computers] could affect Japan's ability to tackle the Y2K problem" (the 'millennium bug'), says a spokesman from Toshiba, a Japanese company that

makes its semiconductors in Taiwan.

Although the earthquake had a substantial effect on Taiwan's industry, research at universities and research institutes seems to have escaped. Hsieh says that 8,000 private researchers at the Hsinchu Science Park have resumed their activities, and that the two universities near the park were unaffected.

"Some of the institutions in central Taiwan, such as the Agricultural Research Institute, experienced damage to their buildings and equipment, but the overall loss is likely to be only around NT\$100 million, and their core research activity is unlikely to be affected," says Hsieh.

Meanwhile, seismologists and engineers from around the world are flocking to Taiwan to analyse the impact of the earthquake and to assess the structures of the collapsed buildings.

The earthquake on 20 September, which measured 7.7 on the open-ended Richter scale at the epicentre in central Taiwan, caused widespread damage, destroying roads and buildings and depriving people of electricity, telephone and water for days.

As over 1,000 aftershocks ricocheted across the country, more buildings — mainly new high-rise apartment blocks — collapsed, revealing substandard construction that did not comply with Taiwan's strict earthquake building codes.

This failure is being blamed on the construction boom that accompanied Taiwan's rapid economic development over the past decade. Many of the damaged buildings had their structures above the lower levels intact, implying weak, shoddy construction at the base.

According to Hiroyuki Kameda, professor of earthquake engineering at Kyoto University, Taiwan had toughened its building rules following the earthquake in Kobe, Japan, in 1995. But buildings completed during the earlier construction boom were not required to meet such standards. **Asako Saegusa**

Scientists wary of weapons research agency

Washington

Concern is growing at the US Department of Energy's laboratories that the planned creation of an agency to run their nuclear weapons research will undermine other programmes, including \$3 billion worth of scientific research each year.

The laboratories, which employ 30,000 people, conduct most of the basic physics research in the United States as well as important programmes in environmental science, biology and other disciplines.

A National Nuclear Security Administration will come into being next March if, as expected, President Bill Clinton signs a defence authorization act that legislates for its establishment, passed by both houses of Congress this month. The agency will be semi-autonomous within the Department of Energy (DoE).

The establishment of the agency is supported by many researchers at the three huge weapons laboratories — Los Alamos and Sandia in New Mexico, and Lawrence Livermore in California. They hope it will free them



Under threat? Los Alamos health research laboratory.

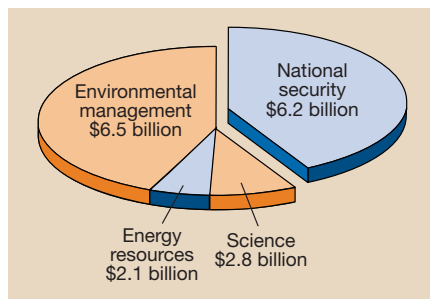
from some of the bureaucracy that has characterized their management by the DoE.

But scientists working on non-weapons programmes in these labs worry that support for their work will erode over time, as the laboratories focus on the new agency's mission of nuclear weapons research. Each of the three laboratories receives annual funding of around \$1 billion, of which one-third at Livermore and one-quarter at the two New Mexico labs is for non-weapons programmes.

Managers and scientists elsewhere in the department's huge network of laboratories fear that their needs have been overlooked in the reforms, which were rushed through Congress this summer in response to allegations of Chinese spying at Los Alamos.

In the short term, the non-weapons labs fear that support will be undermined for the work they do for the weapons programme. About one-third of the work of the Pacific Northwest National Laboratory (PNNL) in Washington state and the Oak Ridge laboratory in Tennessee serves missions that will be transferred to the new agency. But both laboratories will remain under the direct control of the DoE.

"We don't think that, for a change of this magnitude, this has been properly thought



Breaking away: distribution of DoE's budget for 2000, showing money destined for new agency.

through," says Bill Madia, director of PNNL. Madia says the legislation makes it clear that the weapons labs will continue to do science work, but says nothing about the weapons work done by the science laboratories.

This includes \$150 million worth of work at his lab on nuclear non-proliferation, much of it involving safeguarding nuclear facilities in the former Soviet Union. "The question is whether the science laboratories will contribute to the new agency: at the moment, this is very unclear."

Even staff at laboratories that do no weapons-related work are worried about where the reform will leave the energy department in the longer term.

Once the \$6 billion worth of weapons work has been transferred to the agency, an unreformed DoE will be left with \$6.5 billion to cope with the seemingly intractable task of cleaning up old weapons-production facilities, \$2 billion for politically contentious energy-related programmes, and \$3 billion for its science programmes.

The reform "will leave the DoE as a hollow shell, with not much of a budget," says an official at one of the physics laboratories.

With weapons research in the new agency, the DoE's many enemies in Congress

will be better placed to attack the rest of the department's budget. "It's going to make it easier for Congress to abolish the department," says Steve Dean of Fusion Power Associates, a fusion-research advocacy group, and a long-time observer of the department.

Representative William Thornberry (Republican, Texas) framed the reform legislation setting up the new agency along with Senator Pete Domenici (Republican, New Mexico). Thornberry admits that the interests of the nuclear weapons programme came first. "For me, the most important mission of the department is the maintenance of a safe and reliable stockpile of nuclear weapons," he says.

He adds that it was not feasible for the legislation to reform the rest of the department. Thornberry, and congressional staff involved in writing the legislation, say that scientific exchange should continue between the weapons and the non-weapons labs. But they say it is up to the Department of Energy to work out how this should be done, as it implements the law over the next few months.

Energy secretary Bill Richardson has opposed the reforms and called on Clinton to veto them. The president is unlikely to do so because of the margins by which the law passed. So the DoE is starting quiet preparations to implement the reforms and is said to have begun sounding out candidates to run the agency.

Environmentalists are perturbed by the autonomy to be granted to the agency. They sought to derail the agency proposal in Congress on the grounds that it would weaken environmental controls in the nuclear weapons complex. Backers of the reforms counter that the agency will have to adhere to existing environmental laws. **Colin Macilwain**

Allègre defends synchrotron plans

Paris

Claude Allègre, France's science minister, has publicly outlined his reasons for scrapping plans for a French synchrotron, and instead joining British plans to build a similar machine.

Arguing that money spent on large, 'Big Science' facilities should be rerouted into research projects, Allègre said in a written statement last week that to do this France had to team up with other European countries to build such facilities.

But political opposition to his decision remains strong. At a speech to a committee of the Academy of Sciences, president

Jacques Chirac denounced Allègre's decision. "The Soleil project is one of the best scientific facilities that France can realize in the years to come with its scientific community for research and progress, notably in the sectors of health and industry," said Chirac.

The staff at the LURE synchrotron, at Orsay outside Paris, who have been refusing to switch on their facility's 800 MeV and 1.85 GeV machines for the last three weeks in protest, demonstrated last week at the regional council of the Ile-de-France, which lobbied the government to host the facility. They were backed by the general council

AP
of the department of Essonne.

In his statement, Allègre says that "to preserve the possibility of realizing other, more urgent,

projects, we could have simply decided not to finance the synchrotron project, seen as too expensive and not urgent. We did not do that. The strategy adopted was to preserve the interests of the French scientific community, in particular the users of synchrotron radiation, in searching for a European solution.

"Are we in the future going to be lacking synchrotron time? Nothing indicates that. Of course, certain administrators and some users of these machines say so, and without doubt believe it, but experience teaches us that in this domain it is difficult to plan ahead."

To boost research labs, emerging disciplines and the recruitment of young scientists, Allègre says he has decided to rebalance the budget by decreasing the funds spent on large facilities. To accomplish such a reduction, Allègre says his aim is to make any new, large facilities European.

The minister adds that he chose Diamond, the planned 3-GeV machine, because FF1.1 billion of the FF1.8 billion (US\$286 million) needed will be paid by the Wellcome Trust, whereas the French and British governments will each pay FF350 million.

Scientists at LURE challenge some of his figures, arguing, for example, that Allègre underestimates the X-ray needs of French researchers. In contrast to Allègre's statement that French machines are not "saturated", they argue that LURE accepts only 65 per cent of requests for beam-time.

The international synchrotron community has supported the protests, backing the claims of French scientists that they were largely ignored in the decision-making process. Among those behind LURE's cause are the British Nobel Prize winner Max Perutz and the directors of numerous European synchrotron facilities.

But France will only build Soleil if opposition emerges within Allègre's ruling Socialist party. Some members of the party have been critical, but prime minister Lionel Jospin has yet to make a statement on the matter. **Heather McCabe**



Chirac: spoke out for Soleil.

cDNA sequence databank will safeguard research access

Phoenix, Arizona

The US National Institutes of Health (NIH) is about to announce details of the creation of a repository for full-length human complementary DNAs (cDNAs), which will provide a source of both genetic sequences and clones for any researcher who requires them.

The Mammalian Gene Collection, which will eventually also include full-length mouse and rat cDNAs, is designed to bring together molecular data stored by individual researchers. The collection, which federal officials say has been allocated total funding of \$10 million, hopes to have its Internet website functioning next month.

Organizing efforts are being led by the collection's co-directors, Robert Strausberg, assistant to the director of the National Cancer Institute, and Elise Feingold of the National Human Genome Research Institute. Officials say that support for the collection is coming from 16 of the NIH's institutions and the National Library of Medicine.

"We are building this as a resource where anyone can get clones and all information will be available to anyone," said Strausberg. The public storage and availability of such data is being undertaken in part to combat some restrictive corporate efforts to withhold results for proprietary reasons. A full description of the Mammalian Gene Collection will appear in an article in *Science*.

The principal goal of the collection is to

expand genetic storage from expressed sequence tags (ESTs) to full-length cDNA, said Strausberg, who revealed the plans for the repository last week at a meeting on microarrays sponsored by *Nature Genetics*.

More than a million ESTs are stored in GenBank, a repository operated by the National Center for Biotechnology Information. Some ESTs correspond with known genes, whereas others represent partially sequenced novel genes. Providing molecular biologists with a full cloned sequence will give researchers an expanded canvas of genetic material to work with, while ensuring wide access to the crucial information.

The ever-growing need for more molecular depth was evident at the meeting in Phoenix, where about 400 molecular biologists heard presentations on new techniques and discoveries. With microarray technology, researchers can profile the expression pattern of tens of thousands of genes in a single experiment by arraying DNA targets on glass slides or membranes and probing them with fluorescent or radioactively labelled cDNA.

As well as using microarray technology for fundamental discoveries, researchers are broadening applications into disease diagnosis and plant biology. The technology is being used to try to determine the molecular cause of diseases, helping to define new classifications of diseases and redirect treatments for some malignancies. **Rex Dalton**

Fur flies over rare-species panel

Montreal

Canadian biologists and environmental groups are angry at what they claim are continued efforts by provincial and federal governments to politicize the process by which species are added to the endangered list. In particular, they are worried at moves to give representatives from private corporations direct involvement in the process.

Earlier this year, more than 600 scientists wrote to the prime minister, Jean Chrétien, demanding that the rights to identify and list endangered species be given exclusively to scientists, and that politicians should not be allowed to override such decisions.

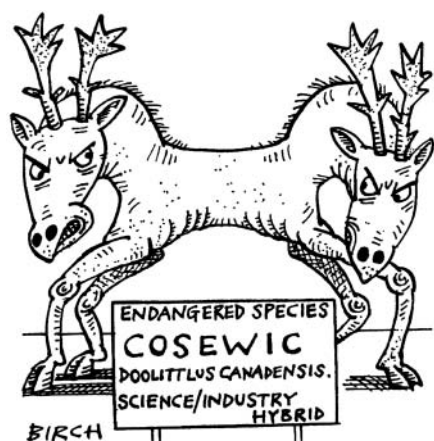
Christine Stewart, then Canada's environment minister, responded by adding eight more scientists as voting members to the Committee on the Status of Endangered Wildlife in Canada (COSEWIC), which identifies the species that should be on the list (*Nature* 398, 9 & 358; 1999). The govern-

ment had previously removed the voting rights of most non-governmental scientist members.

But a draft operating manual for the committee, leaked to *The Globe and Mail* newspaper in Toronto, shows that, for the first time, members of private corporations would be allowed to sit on the committee.

Committee member Gilles Seutin of McGill University says that many members are concerned that the government is trying to dilute its decisions. And David Schindler of the University of Alberta, who drafted the earlier letter to the prime minister, says there is no place for non-scientists, politicians or industry representatives on COSEWIC. Schindler cites the possibility of a biologist working for a forestry company that might have an interest in not seeing a particular bird placed on the list.

According to the newspaper report, the leaked document also says that COSEWIC



would no longer be able to release its endangered species list independently, but could only do so jointly with federal and provincial wildlife ministers, and the new Canadian Endangered Species Conservation Council.

Sarah Dover, director of the Canadian Endangered Species Campaign for the Canadian Nature Federation, says that, because the council is a political body, "there could be political interference".

But David Brackett, director-general of the federal Canadian Wildlife Service, maintains that adding corporate representatives would not interfere with the committee's independence.

David Spurgeon

Spanish university chiefs blast recruitment system

Barcelona

A survey by Spain's Council of Universities has revealed widespread dissatisfaction among university heads with the procedures for faculty appointments (see *Nature* 396, 709 & 712; 1998).

The results of the survey were revealed by Jorge Fernández-Díaz, state secretary of education and universities, during a meeting in Santander this month on the future of university government.

The survey polled the Council of Universities, whose members include university vice-chancellors, heads of regional education departments, and 15 other members and presidents of the Social Councils — bodies that supervise university departments — about recruitment, funding and the performance of university governing bodies.

University appointment boards have five members, two of whom belong to the recruiting university. Three votes are needed to approve an appointment, and most observers — including government and university officials — agree that it is usually easy to secure the third vote for a favoured local candidate.

Of those surveyed, 84 per cent disagreed

that "the system of access to professorial posts is appropriate for an effective selection of research and teaching personnel". Asked about the composition of the evaluation panels, most said that the number of members chosen by lot should be increased.

Fifty-eight per cent said that at least four members should be chosen this way, and seven per cent said all five members should be chosen by lot. Only eight per cent thought the current system should be maintained.

One in five said that there should be an increase in the number of members designated by the university at which the appointment is being made, and 15 per cent said that all members should be designated by the university.

Leonardo Sánchez-Ferrer of the Universidad Complutense de Madrid, who carried out the survey, says that demands for greater autonomy in universities are traditionally louder in nationalistic regions such as Catalonia, which could lead to resistance to change.

Mariano Rajoy, minister of education and culture, told the Santander meeting that "a profound and integral change" is needed in the university reform law.

Xavier Bosch

Geographer sues critics of his rock-dating methods

San Diego

An Arizona State University (ASU) geographer under federal investigation for possible scientific misconduct is suing the authors of an article in *Science* that claimed that the geographer's rock-dating methods were fundamentally flawed.

According to court records, Ronald Dorn contends that the authors "intentionally manipulated, omitted and misrepresented data for the purpose of making it appear [Dorn] engaged in professional misconduct and impropriety". The authors include leading Earth scientists from the University of Arizona and Columbia University.

The article reported that 80 of Dorn's samples of rock varnish contained what appeared to be pieces of coal and charcoal-like material (see *Science* 280, 2132–2135; 1998). Mixing these substances — which have widely differing radiocarbon dates — can produce any desired date for a sample.

Dorn's dating method involves scraping varnish off rocks and employing radiocarbon labs to date samples from geological formations and rock art sites.

Some authors of the *Science* article first reported suspicions about Dorn in 1996 to

the Office of the Inspector General of the National Science Foundation (NSF), which funded much of Dorn's research on the questioned samples of rock varnish (see *Nature* 392, 218–219; 1998).

ASU has completed its investigation of Dorn, according to a university official, and sent its findings to the NSF's inspectors, who will determine whether any misconduct occurred. Neither ASU nor NSF officials would disclose the university's conclusions, nor comment on the probe.

Dorn filed his lawsuit on 25 June, a day before the statute of limitations would have expired for filing a complaint about the *Science* article. Dorn's attorney, Terry Hall, did not respond to interview requests.

Dorn's lawsuit was filed against Warren Beck, Douglas J. Donahue, A. J. T. Jull and George Burr, of the University of Arizona's NSF-funded Accelerator Mass Spectrometer Laboratory; Wallace S. Broecker of the Lamont Doherty Earth Observatory at Columbia University, which is also a named defendant; and Ekkehart Malotki of Northern Arizona University.

Dorn also sued the wives of the scientists, in an apparent attempt to

maximize potential economic recovery and pressure. But the lawsuit does not name Eidgenössische Technische Hochschule, the Swiss institution whose staff contributed to the *Science* article.

Tommy Thompson, the University of Arizona's attorney for that campus's authors, declined to comment, as did those of the authors contacted by *Nature*. The authors have denied previous contentions by Dorn that they manipulated results to impugn him. Court records show that Dorn's attorney has not served the authors with the lawsuit, which officially starts the court process before a judge. The attorney must serve the defendants within 120 days from 25 June or risk dismissal of the case.

Dorn is suing for defamation, intentional infliction of emotional distress and interference with prospective economic advantage. In the latest twist in the case, on 21 September Dorn's attorney filed a claim — a prelude to a lawsuit — against the University of Arizona and its authors, alleging that they mislabelled, confused or contaminated 96 rock samples submitted for radiocarbon dating. Dorn is seeking \$482,542 in damages.

Rex Dalton

OECD finds France and Sweden spend most on knowledge

Paris Sweden and France top the list of industrialized countries that invest the greatest proportion of their gross domestic product (GDP) in 'knowledge' — defined as including education, software and research spending. So says the latest report from the Organization for Economic Cooperation and Development (OECD) in Paris.

The statistics have been compiled to reflect growing political interest in 'knowledge-based economies'. They show that each of these countries invests almost 10 per cent of its GDP in these three activities, compared to an OECD average of 8 per cent (private-sector spending is not included).

The report, *OECD Science, Technology and Industry Scoreboard 1999*, points out that this is comparable to levels of investment in physical equipment. Public spending on 'intangibles' in the United States is slightly above the OECD average. Italy and Japan spend least — between six and seven per cent.

"These figures represent the first international snapshot of how much countries invest in knowledge, and represent a more sophisticated measure than simple inputs such as research and development

expenditure," says an OECD official. The countries following Sweden and France at the top of the list are Denmark, Finland and Norway.

High-resolution satellite takes to the sky

Washington The first commercial satellite capable of returning spy-satellite-quality images of the Earth was launched last week from Vandenberg Air Force Base in California. The IKONOS satellite, owned by Space Imaging of Denver, Colorado, takes panchromatic pictures with one-metre resolution and multi-spectral images with four-metre resolution, and can rephotograph the same location every five days. Images will be on sale within 90 days, with prices starting at \$75 per square mile.

Atmosphere is right for US and China

Beijing China and the United States announced last week that they will continue with a cooperative programme on atmospheric research resulting from an agreement signed by former US President Jimmy Carter and the late Chinese leader Deng Xiaoping in May 1979, the first between China and the United States after the normalization of diplomatic relations.

More than 1,000 scientists and professionals from the two countries have since been involved in the programme. The two sides have agreed that future cooperation will focus on climate and monsoon research, atmospheric chemistry, satellite meteorology, mesoscale meteorology, tropical cyclones, meteorological modernization and training and participation.

Council of Europe says no to patenting genes

Munich The parliamentary assembly of the Council of Europe last week approved a recommendation that "neither plant, animal nor human derived genes, cells, tissue or organs" should be considered as inventions, and thus become the possible subject of patents.

The move is in conflict with a European Union directive approved last year, which allows the patenting of transgenic plant and animals. But the assembly warns that patents on living organisms could threaten biodiversity, particularly in developing countries. It calls on the governments of the 41 members of the council to discuss a "suitable alternative system of protecting intellectual property in biotechnology" consistent with the 1992 United Nations Convention on Biological Diversity.

US genome institute plans new centres

Phoenix, Arizona The US National Human Genome Research Institute is planning to fund a number of new centres to conduct multidisciplinary research on genetics. Competitive proposals for planning grants for 10 to 20 new centres are expected to be announced in the next few weeks, according to Francis Collins, the head of the institute.

Collins estimated that each centre, which might be based at one academic institution or incorporate researchers from several nearby universities, could require a couple of million dollars a year to operate. The funding will provide the centres with "the opportunity to acquire the core technologies that are expensive and required to technically support" current genetics research, Collins said. He added that other NIH institutions are expected to fund projects undertaken by the new centres.

Japan and Australia to lay underwater cable links

Tokyo Telecommunications companies from Japan, Australia and Canada are planning to set up a joint venture to lay high-capacity marine fibre-optic cables linking Japan and Australia via the island of Guam. The cables

are planned to become part of a wider network of high-capacity cables between Japan, the United States and Southeast Asia to meet growing demand for data communications between the regions.

Japan Telecom, Australia's Telestra and Canada's Teleglobe plan to invest a total of ¥53 billion (US\$495 million) in the initiative to lay the 10,000-kilometre cables. The joint venture will be called the Australia Japan Cable System. The cables, which will be the first high-capacity link between the two countries, are expected to become operational by the end of 2001.

Telescope sent to sea to catch neutrinos

London Work has started on the installation of a telescope at the bottom of the Mediterranean to detect and study high-energy cosmic neutrinos. The £10 million (US\$16 million) telescope, Antares (Astronomy with a Neutrino Telescope and Abyss environmental Research), will be located 40 kilometres southeast of Marseilles, France. It will lie 2.4 km below the sea's surface.

Antares, part of an international effort to detect neutrinos, will detect light radiation from the occasional interactions between neutrinos and sea water. Neutrinos, which pass almost unhindered through all forms of

matter, are of interest to both the astrophysics and particle-physics communities. The initial string of detectors was deployed last week, the first step towards a much larger and more sensitive telescope that is planned to be in place by 2002.

Fertility researcher blasts UK as he departs for Canada

London A leading British fertility researcher last week blamed the reluctance of the British government to approve the experimental cloning of human embryos for purely therapeutic purposes as an important factor in his decision to take up a post in Canada.

Roger Gosden of Leeds University revealed his departure plans shortly after the public announcement of his successful development of a technique for removing and subsequently reimplanting female ovaries. His work has been hailed as a major breakthrough of potential benefit to, for example, young women required to undergo radiotherapy.

Gosden argued that criticism of biotechnology in Britain has helped create a climate of antagonism towards research of potentially important therapeutic value. "With all the fuss over genetically modified food and so on, it is difficult to be a scientist in Britain," he is reported as saying.

Online archive must serve authors as well as publishers

Sir—In an extremely important, timely and welcome development for science, the E-Biomed proposal has evolved into PubMed Central, a free online public archive of the peer-reviewed and non-peer-reviewed literature in biology. It will be launched by the US National Institutes of Health (NIH) next January (*Nature* **401**, 6; 1999). There is only one fundamental question that needs to be answered about the revised proposal: will authors be able to self-archive their refereed articles in PubMed Central?

The revised proposal is not clear about this question: it could be that only publishers will be able to archive refereed papers. This would be regrettable, because publishers are not likely to want to give away papers free, whereas authors are.

If authors are allowed to self-archive their refereed articles, PubMed Central will not only quickly make the biological literature into the optimal free resource for biological science, but it will provide a model for adoption by all other learned disciplines.

The director of the NIH, Harold Varmus, says that PubMed Central will be a web-based repository for barrier-free access to primary reports in the life sciences. Assuming that "barrier-free" means free for one and all in perpetuity, this will be an invaluable contribution to the advancement of biological and medical research.

Varmus also says that the screening of non-peer-reviewed reports will be the responsibility of groups that have no direct relationship to the NIH. This is as it should be. Peer review should continue to be implemented by scientific publishers and societies, and reports should be provided to PubMed Central from participating publishers and societies that have mediated the review process. But what about peer-reviewed reports from non-participating publishers and societies? Will the authors of such work be able to archive it in PubMed Central too? Or will the work available for free for all in PubMed only be that published by 'participating' publishers and societies?

The non-peer-reviewed reports will also enter PubMed Central through independent organizations, which will be responsible for screening this material. Will authors be able to self-archive their non-peer-reviewed reports? Some screening is a prudent idea but it must not be so restrictive as to prevent the self-archiving of preprints that are being submitted to peer-reviewed journals.

Will the availability of the peer-reviewed literature online free for all be conditional only on the active collaboration of publish-

ers (who currently derive their revenue from selling it) or also on the active collaboration of authors (who give it away)?

Varmus has asked publishers, societies, editorial boards and other organizations interested in depositing content in PubMed Central to contact him at PubMedCentral@nih.gov. But what about authors interested in depositing their peer-reviewed and non-peer-reviewed reports? Is, say, university affiliation sufficient (which would be a good first step), or is it still to be only publishers who determine whether or not their authors' freely given reports can be given away for free? A great deal rests on the answer to this question.

It is to be hoped that, as PubMed Central accrues more and more of the literature and makes it available to everyone for free, the bioscience community will become as addicted to this online archive as the physics community has become to the Los Alamos archives. In that case, the freeing of the rest of the literature will not lag far behind.

An online debate on this topic is at <http://amsci-forum.amsci.org/archives/september98-forum.html>

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A view from Kansas on that evolution debate

Sir—I have recently attended two lectures in the wake of the controversial decision by the Kansas State Board of Education to "eliminate" the required teaching of evolution (see *Nature* **400**, 701; 1999). Philip Johnson, a professor of law at the University of California, Berkeley, and John Staver, co-chair of the committee responsible for drafting the new Kansas standards—and whose draft had been, according to him, "severely edited" by the board to "remove evolution"—both presented their definitions of science and evolution to sympathetic audiences. Both erroneously presented what they believed to be the other party's definitions of these concepts.

The crucial difference between what the creationists believe and what the proponents of evolutionary theory accept concerns the issue of whether the origins of life were driven by randomness or by an intelligent creator. Many creationists are supportive of scientific enquiry for biblical reasons such as in Romans 1:20, "For since the creation of the world God's invisible qualities, his eternal power and divine nature, have been clearly seen, being understood from what has been made".

Creationists, according to Johnson, do not doubt that DNA encodes the features of

an organism or that changes in DNA (mutations) give rise to variation in those features which are subject to selective pressures in nature. Mainstream creationists also accept that genetic and phenotypic changes could result in speciation. They consider evolution as a plausible model to account for the natural history of living things, but they see a great distinction between the empirically proven elements of evolution (micro-evolution) and the explanation of speciation and origins of life (macro-evolution). Students in Kansas will still be required to learn the former, but it will be left to local school districts to decide whether they are required to learn the latter.

The lesson to be learned from the events in Kansas is that science educators everywhere must do a better job of teaching evolution. It must be made clear that the evidence supporting the mechanism of evolution is empirical and proven, but that speciation and natural history are derived from the admittedly weaker evidence of observation. The fact that one cannot reproduce the experiment does not diminish the validity of macro-evolution, but the observed phenomena supporting the theory must be presented more clearly.

Additionally, one must question the interpretations of the observed phenomena and discuss the weaknesses of the model. Honest scientists are far more inspiring than defensive ones who scoff arrogantly at the masses and fear that discussing the problems of macro-evolutionary theory will weaken general acceptance of it. On the contrary, free debate is more likely to encourage the curious to seek solutions. Most important, it should be made clear in the classroom that science, including evolution, has not disproved God's existence because it cannot be allowed to consider it (presumably).

Even if all the data point to an intelligent designer, such an hypothesis is excluded from science because it is not naturalistic. Of course the scientist, as an individual, is free to embrace a reality that transcends naturalism.

Scott C. Todd

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Wellcome for education on science in society

Sir—You have reported criticisms of the new science centre, Explore at Bristol (*Nature* **400**, 801 & 804; 1999; see also response from Gillian Thomas, *Nature* **401**, 111–112; 1999). There is currently a great deal of public criticism about aspects of medical research which needs to be addressed by those involved. The

Wellcome Trust spends about £400 million (US\$640 million) a year on research, and has committed roughly £34 million of this to science centres and museums in the United Kingdom, including Bristol. Why?

It is crucial to explain to the public the excitement of medical research and its potential benefits without ignoring its social impact and implications. The Wellcome Trust supports initiatives such as Explore at Bristol precisely because they aim to go beyond the traditional 'hands-on' approach to explore the wider context within which biomedical science develops.

Traditional 'hands-on' science centres barely touch modern science or biomedicine, and the social context is ignored altogether. Although hands-on exhibits are powerful learning tools, they are not necessarily the best way to tackle modern biology or the social issues raised by the human genome project, for example. Yet the general public has to be informed about the place of science in society if it is to trust science and scientists.

This task is by far the most important facing the scientific community at present and the new science centres will be crucial in this regard. There may indeed be teething troubles, not least owing to the very short timescales over which these huge projects have had to develop. But the Wellcome Trust's interest is long term, not only helping to build on the solid foundations of past innovation, but also aiming to experiment with new directions.

Laurence Smaje

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Confidentiality is vital to bioweapons control

Sir— Biological weapons still form part of the world's arsenal, as the work of the United Nations Special Commission (UNSCOM) in Iraq has underlined¹. A weapons control system based on UN Security Council resolutions implies serious threats not just to national sovereignty but also to confidential information — an important concern in today's competitive academic and industrial environment. Since a treaty-based inspection regime relies on the willingness of states to sign the treaty, they have to be certain that their confidential information will remain secure.

Current negotiations in Geneva for a protocol to strengthen the biological weapons convention² are likely to lead to a combined reporting–inspection system. As in the chemical weapons convention³, site inspections and inclusion of non-military sites are being discussed: two elements with

far-reaching consequences for confidentiality issues. In addition to classical confidentiality provisions (such as guidelines, a commission, individual secrecy agreements, and restricted access to information), the selection of appropriate triggers for countries to make declarations under the protocol should weed out threats to the loss of confidential information.

The draft protocol confines required declarations of research and development to listed agents and toxins, biological defence and maximum containment facilities. The current talks are still discussing the scope of inspections and when they should be held² — for example, should they be random? Inspections will be based on mandates defining the purpose of the inspection, ranging from confidence-building, auditing and clarifying information to investigating a suspected breach of the biological weapons convention.

Until now, concerns about the risk to confidential information have not been substantiated by declarations based on the current draft protocol and several practice inspections.

The trend to incorporate private institutions can also be seen in other international agreements, such as the Convention on Access to Information, Public Participation in Decision-making and Access to Justice in Environmental Matters⁴ and the draft Biosafety Protocol to the Convention on Biological Diversity⁵.

In order to increase public and political acceptance of biological research and biotechnology, confidentiality issues and the need for more transparency will have to be brought into balance.

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Devil in the detail

Sir— William Thompson [*sic*] and Lord Kelvin are credited with naming Maxwell's demon in Seth Lloyd's obituary of Rolf Landauer (*Nature* **400**, 720; 1999). The demon, or at least an editorial gremlin, is having a little joke: William Thomson (without the 'p') and Lord Kelvin were one and the same, transmuted by act of Queen Victoria in 1892.

Nicholas J. Cox

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Turning the tide



View from the Rock:
how it should look.

Sir— A cartoon in News and Views seems to prove that 5.33 million years ago the Mediterranean emptied into the Atlantic rather than filled from the Atlantic (*Nature* **400**, 613; 1999). Must we reverse our theories, or should you reverse your slide?

Tim Robinson

*Folding Landscapes, Roundstone,
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Medicine and biology are more than biomedicine

Sir— While I concur with Ken Dill's call for increased support of research in physics, chemistry, mathematics and computer science, I am troubled by his reasoning¹. He conflates biology and medicine into an ill-defined hybrid "biomedicine", which he believes is reducing "the problems of disease to problems of molecular science". I believe this belittles both biology and medicine.

The biological sciences are quite distinct from medicine. Obviously they overlap, but so do each of them with the other disciplines that Dill mentions. Further, advances in biology and medicine feed back into and stimulate what he terms the "basic sciences", and may lead to whole new research paradigms².

The unidirectional model of "basic" and "applied" research implied by the pyramid in his Fig. 1 derives less from his view of their interactions than from the rapid growth of the budget of the US National Institutes of Health (which supports "biomedicine") compared with US agencies concerned with the physical sciences.

Many research programmes could influence human health and need new, long-term funding. Among these, I would stress patient-oriented medical research itself³, including human pathophysiology, epidemiology, pharmacology, clinical trials and health-services research. A good case could also be made for behavioural, population and other as yet 'soft' sciences. If Dill wishes to present a two-dimensional model for the relationships of the sciences to the curing of disease, I would suggest a circle with radiating spokes for the many disciplines that need increased support.

Alan N. Schechter

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Germany's forgotten war

Nazi Germany also fought against environmental and industrial carcinogens.

The Nazi War on Cancer

by Robert N. Proctor

Princeton University Press: 1999. 380 pp.

\$29.95, £18.95

Ute Deichmann

In 1944, at the height of the Second World War, the influential German surgeon and cancer researcher Karl Heinrich Bauer compared the continued use of the synthetic dye known as butter yellow, which had been known to be carcinogenic for some years, to a "human experiment on a massive scale". This was not a trivial pronouncement. Bauer was one of many leading physicians and health politicians in Nazi Germany who warned of the dangers of carcinogens and other environmental health hazards.

The Nazis recognized this issue earlier than did authorities in other countries. Robert Proctor, by focusing on medical research and public-health policies that today are regarded as progressive, reviews a neglected aspect of the history of medicine in the Nazi era. This contrasts with other recent studies that emphasize the involvement of the medical profession in racial crimes, including the large-scale experiments on humans, which Proctor examines in his earlier book *Racial Hygiene: Medicine Under the Nazis* (Harvard University Press, 1988), and by no means ignores here.

In his well-documented and highly readable book, Proctor deals with the central aspects of Nazi health policy, previously unknown environmental cancer experiments, fights between industry and Nazi health activists, and the warped value system of physicians with regard to healthy Germans and the 'racially unfit'. The Nazi war on cancer was largely based on prevention, including early detection, improved diet and anti-tobacco campaigns, rather than on curative measures.

Germany had a strong tradition of research into occupational cancer, and had passed protective legislation in the late nineteenth century. Thus, cancer caused by certain intermediates used in the German aniline (synthetic or coal tar) dye industry around 1900 was studied intensively because Germany was the world's largest manufacturer of synthetic dyes. In 1926, Germany was the first nation to compensate uranium miners who developed lung cancer.

The identification of workplace carcinogens remained a central issue of the Nazi cancer programme. 'Race hygienists' warned about possible genetic damage from X-rays, opposing professional radiological societies. In 1938 several German publications provided



Two killers: in contrast to that on other fronts, the Nazi war on cancer was mainly one of prevention.

ed strong evidence for the link between asbestos and lung cancer. Interestingly, the researchers did not rely on epidemiology (as did scientists in the United States two decades later), but on clinical and pathological insights, using the newly developed electron microscope. This evidence led the Nazi government to be the first to recognize asbestos-induced mesothelioma and lung cancer as compensatable occupational diseases in 1943. Despite mandated protective measures since 1925, new cases of bladder cancer (by the 1920s suspected of being caused by β -naphthylamine and benzidine) continued to appear in the dye industry.

Inevitably, the focus on production and the rapid build-up of the arms industry shifted Nazi labour leaders' attention away from the hazards of cancer. And with the policy of employing foreign and slave workers, efforts to eliminate illness were replaced by efforts to eliminate sick workers, as was the practice at IG-Farben's Auschwitz plant. The efforts of industrial hygienists to identify people genetically predisposed to tumours, part of the strategy of selecting workers without such a predisposition for hazardous activi-

ties, were eventually sidelined when foreign and slave workers were given this work.

Public-health policy was directed exclusively towards the protection of non-Jewish Germans, sometimes only as long as they were still of economic use. The racial state was the motivation for all progressive health measures. Thus, environmental measures were furthered by the obsession of Hitler, Himmler and Hess with food and health. Other Nazi leaders were among the chief anti-tobacco lobbyists, in particular Fritz Sauckel, the SS activist and gauleiter of Thuringia notorious for his brutality in the organization of foreign labour; Reich health fuehrer Leonardo Conti, one of the organizers of the euthanasia operation; and Karl Astel, the SS activist, racial propagandist and professor of anthropology at the University of Jena. Sauckel was executed in 1946 after being convicted at the Nuremberg trials of war criminals. Astel and Conti committed suicide before they were brought to trial.

Nazi Germany had the world's strongest anti-smoking campaign. It was the first country to establish a link between smoking and lung cancer based on clear epidemiologi-

cal evidence. Franz Hermann Mueller, in his medical dissertation at the University of Cologne in 1939, presented the world's first controlled epidemiological study of the link between smoking and lung cancer, in what Proctor describes as an "exquisite piece of scholarship". Mueller's study was little noticed outside Germany, and his fate is unknown.

A more comprehensive study was done in 1943 by Eberhard Schairer and Erich Schoeniger at the Jena Institute for Tobacco Hazards Research. This institute was founded by Astel in 1941 with the help of a grant of 100,000 reichmarks from Hitler's Reichskanzlei, and Astel made Jena the centre for anti-tobacco activism, banning smoking from all university and health offices.

The battle between the tobacco industry and Nazi criminals acting as health activists is a bizarre episode. In particular, the tobacco magnate Philipp Reemtsma in Hamburg waged a 'miniwar' against the regime; the Nazi attacks ceased after Reemtsma gave several million reichmarks to Goering. Counter-attacks by industry against the anti-smoking campaign led to an alliance between the anti-tobacco activist Conti and the Ministry of the Interior. The Ministry of the Economy backed the tobacco industry, and Goebbels backed Conti.

The tobacco industry seems to have lost the public-relations battle, but it is unclear to what extent sales were affected. Proctor acknowledges support for his research from Reemtsma's son Jan Philipp, who has spent millions of deutschmarks since the 1980s on research and campaigns to inform the German public about Nazi crimes.

Proctor intentionally does not deal with scientific cancer research other than research into carcinogens. Therefore, his claim that "the Nazis were pioneers in cancer research", or that "quality science" not only continued in Nazi Germany but was even initiated as an outcome of Nazi ideology, should be considerably qualified. There is evidence that, despite heavy funding and the involvement of internationally renowned researchers, there were few scientific successes in areas of basic cancer research, particularly in biochemistry.

Proctor believes that his study will lead to a revision of traditional viewpoints on the impact of National Socialism, in particular that it will be viewed as a more subtle and plausible phenomenon than we commonly imagine. It is true that National Socialism had many advantages for most non-Jewish and healthy Germans. This is clear from interviews with those who lived through the Nazi era in Germany, and who even today tend to emphasize the reduction in unemployment and crime, the network of autobahns, publicly financed vacations for needy children and new national self-esteem and pride.

Based on new and challenging findings about the success of the Nazi environmental

cancer science and health policy, Proctor argues, and I believe with considerable justification, that today's public-health measures, preventive medicine and anti-smoking campaigns should not be belittled because the Nazis were among the first to introduce them. At the same time, this book clearly shows that the existence of some good science and powerful public-health measures does not render the Nazi regime and many of its medical scientists any less murderous. It should not be forgotten that the context of the beneficial health measures was nothing less than that of a murderous racial policy. But this is an important book which will encourage the reader to reflect on the ways in which medical science was conducted and used in the twentieth century. ■

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..... Feynmania

The Pleasure of Finding Things Out

by Richard Feynman

Perseus Books: 1999. 320 pp. £18.50

Graham Farmelo

Woody Allen was once my *bête noire*. It wasn't his fault: although I had neither met him nor seen any of his films, I irrationally took against him in response to all the hype lavished on him. And, I am ashamed to say, I felt pretty much the same about the late Richard Feynman.

It took me several years to overcome this aversion and to distinguish Feynman the great physicist from Feynman the beguiling showman. The quality of his science speaks to us through his great theoretical work on subjects ranging from quantum electrodynamics to the phase changes of liquid helium. He may not rank with Einstein, Rutherford and Fermi in the pantheon of the century's greatest physicists, but there is no denying that he was blessed with a truly brilliant intuition. This ability, together with a steam-hammer mathematical power — deployed only when the occasion demanded — made him a unique talent.

As if this were not enough, he was also a colourful character, and the physics community has long traded stories of his adventures, intellectual and otherwise. A lively collection of his favourite anecdotes, as told to his friend Ralph Leighton, were published in the surprise 1985 best seller *Surely You're Joking, Mr Feynman* (W. W. Norton, 1997), which made him one of the few scientist-celebrities in the United States. These stories did much to undermine the stereotype of the physicist as a stuffy, head-in-the-clouds bore, but they left me cold.



The other face of Feynman: at the Challenger Space Shuttle inquiry.

The critical consensus was that the tales were both hilarious and endearing, but I found them at best mildly amusing and not a little irksome, if only because the tiresome point of all of them was that their author was the smartest darned guy you've ever heard of.

Following the success of *Surely You're Joking*, more collections of Feynman stories have made their way into print, and they seem to have become successively thinner. So my heart sank when I came across *The Pleasure of Finding Things Out*, a compendium of 13 articles and transcripts of little-known talks and interviews, all accessible to non-specialists. Were these the dregs of the Feynman legacy?

I need not have worried. Although some of the pieces are weak, there are enough good ones to make the collection worth reading. The book gets off to a cracking start with Freeman Dyson's foreword, in which he lucidly compares his feelings for Feynman to those of the Elizabethan playwright Ben Jonson for William Shakespeare. Jonson wrote that he "did love the man this side idolatry", a phrase that crisply captures Dyson's admiration and affection for his friend and mentor.

Dyson alerts us to several of the essays' themes, especially to Feynman's favourite: the virtue of doubt. For Feynman, the essence of scientific enquiry is to hold that everything is, to a greater or lesser degree, in doubt. He even puts uncertainty at the heart of one of his attempts to define science, which he believed is "the result of the discovery that it is worthwhile rechecking by new direct experience, and not necessarily trusting experience from the past". His argument is so apposite that you wish more popularizers of science would take up this point as an antidote to the common misconception that scientists do nothing more exciting than add to their inventory of facts.

Another recurring theme of the book, which is more repetitive than its editor

Jeffrey Robbins seems to realize, is Feynman's belief that we don't live in a scientific society. Why else, he asks, would astrology and mysticism thrive? For Feynman, science is a uniquely privileged form of enquiry, and he has no truck with those who associate the term with educational research, psychology or sociology.

However, he reserves his most withering disdain for philosophers, who "should learn to laugh at themselves", he says. This is all good knockabout fun, but one wonders, as he lays into Spinoza's "meaningless chewing around", whether he knew what he was talking about. Feynman's style is impressively direct, but you can't help thinking that some philosophers have much to teach us by paying especially careful attention to the meaning of words and ideas. It would have been fascinating to have heard Feynman justify his views in a head-to-head discussion with a philosopher who was his intellectual match.

Feynman is on surer ground when talking about science and technology, and there are two pieces in this collection that show him at his formidable best. The first is the transcript of his visionary 1959 talk, "There's plenty of room at the bottom", in which he set the agenda for what became known as nanotechnology. In this masterpiece of futurology, he points out in straightforward language what everyone else has come to accept — that the laws of science do not prevent the making of machines at the atomic level. In an aside, he notes that "in the year 2000, when they look back at this age, they will wonder why it was not until the year 1960 that anybody began seriously to move in this direction". It's a safe bet that he'll be proved right on that, too.

The other outstanding piece is his report to the Challenger Space Shuttle inquiry. President Reagan set this up after the 1986 disaster and invited Feynman to join the commission, as its only scientist member. He made a vital contribution to the proceedings by ferreting out the true reason for the disaster (the failure of the O-rings), which he explained in an experiment he dramatically carried out on prime-time television. His report was embarrassingly critical of the US space agency NASA, and he had to fight to have it included, as a minority opinion, as an appendix to the final document. It is a pleasure to read and a monument to Feynman's courage, eloquence and ingenuity.

The Pleasure of Finding Things Out is a delightful reminder of Feynman's prodigious gifts. On the book's inside cover, among the encomia to his talents as a communicator and a scientist, the writer John Gribbin asserts that he "was certainly the greatest physicist, and possibly the greatest scientist, to be born in the twentieth century". Certainly the greatest? Just as Feynman would have wished, I have my doubts. ■

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Astronomy's biggest project

Measuring the Universe: The Cosmological Distance Ladder

by Stephen Webb

Springer-Praxis: 1999. 342 pp. £24.50, \$44.95 (pbk)

G. Jacoby

We started thousands of years ago. We are still at it today: we measure distances to stake out property boundaries, to estimate how far ships are from land, and to determine our place in the scheme of the Solar System, the Galaxy and the Universe. It has never been easy, and attempting to measure the size of the largest 'thing' that exists — the entire observable Universe — must be among mankind's most ambitious intellectual quests. Consequently, astronomers require a tremendously broad understanding of the way the Universe works, from the origin of matter and energy to the life cycles of stars. Nevertheless, centuries of effort by many people have come together over the past decade, culminating with observations from the Hubble Space Telescope, so that astronomers can now determine the size and age of the Universe with good accuracy. That story is the subject of this book.

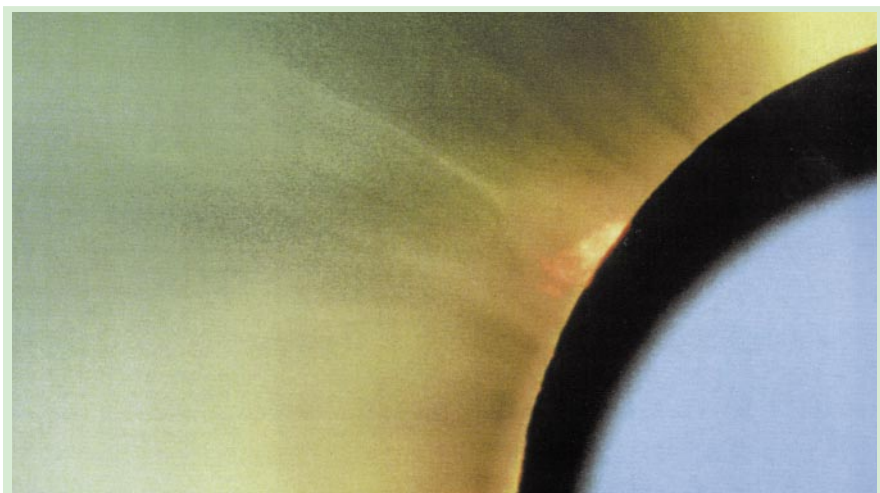
Stephen Webb lucidly presents the scientific basis for deriving distances to the Sun, the Moon, the planets, nearby and distant stars, and to the nearby and the most distant galaxies, despite the necessity of dealing in complex concepts such as relativity, curved space-time and vacuum pressure. His discussion extends from the basic astronomy

of the Solar System up to and beyond the Big Bang, the inflationary period and the resulting cosmic microwave background.

The author adopts a conservative, and perhaps the only, story-telling strategy by beginning with the ancient astronomers and working chronologically to larger distances. I especially appreciated the descriptions of the roles of many of the less celebrated players in the game.

One is tempted to compare *Measuring the Universe* with *The Cosmological Distance Ladder* (1985) by Michael Rowan-Robinson, which is now somewhat dated. Because Webb aims at a broader, less professional audience, his book includes less mathematics and fewer references and data tables than Rowan-Robinson's, and so does not completely displace that book. But Webb is more fun to read, relating many fascinating little tales. There is Fritz Zwicky's definition of a 'sphere' (all extragalactic books require a Zwicky story), the rationale for how the days of the week are named and ordered (which, amazingly, depends on the distance scale!), the story of how mathematics cost Tycho Brahe his nose, and how Giordano Bruno's astronomical beliefs led to his burning at the stake. And, with a bit of poetic licence, Webb uses *Star Trek's* Starship *Enterprise* to view the Solar System from a distance, and has malevolent aliens destroy the Sun to illustrate the travelling time of light (we still see the Sun 8.3 minutes after its destruction).

The mathematics is well aimed at the target audience of beginning science students. Many excellent numerical examples are used to illustrate how today's common observational techniques are applied. Historical and modern references are listed after each chapter; while the bibliography is not complete, it



Solar spectacle

If it's the showier aspects of astronomy that you're after, you can't go far wrong with comets, meteor showers and eclipses. All three astronomical wonders are explained and illustrated in *Cosmic Phenomena* by

Gabriele Vanin (*Firefly*, \$35). The photograph above was taken in Kazakhstan during the 1981 solar eclipse, and shows a bright prominence, together with delicate coronal polar plumes.

samples the breadth of the literature. Also, there are questions for the student; some are easy while others have no answer.

While Webb is nearly objective in reporting the current state of the distance scale, a lot of the public interest in this topic stems from the controversy surrounding it. But Webb gives only limited space to the human element that has fuelled the nearly religious fervour of the distance-scale debate for the past 25 years. His emphasis is probably appropriate, considering the target audience and the availability of Dennis Overbye's *Lonely Hearts of the Cosmos* (Little Brown, 1991). For those seeking an exposé of cosmologists' lives, this is not the book; but, for those interested in an understandable and enjoyable textbook that describes how astronomers measure real distances to real objects that are far away, *Measuring the Universe* hits the mark. ■

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Synchronizing situations

Fast Oscillations in Cortical Circuits

by Roger D. Traub, John G. R. Jefferys and Miles A. Whittington
MIT Press: 1999. 324 pp. \$57.50, £34.95

Dietmar Plenz

The dynamics of groups of nerve cells pose a major challenge in understanding brain function. In that context, *Fast Oscillations in Cortical Circuits* is a refreshing approach to unravelling one of the most intriguing phenomena displayed by the mammalian brain: the γ oscillation. This brain activity, during which neurons synchronize their firing at a rate of approximately 40 Hz, has been found during a vast variety of behavioural states, ranging from REM (rapid eye-movement) sleep to attentive behaviour in human and animal studies.

Traub, Jefferys and Whittington use a combination of neuronal modelling and electrophysiological experiments to outline their ideas on how γ oscillations are generated. This book is not for the novice. Its main focus resides at the minuscule level at which changes in the membrane potential occur in single neurons from a hippocampal slice. The authors show how these changes can be represented by compartmental modelling of neuronal networks. However, the authors also link these cellular-level descriptions to more complex phenomena such as epilepsy, opiate function and memory formation.

The central mechanism proposed to explain γ oscillations is based on the dy-

namics of networks of mutually coupled inhibitory interneurons. At the start of the decade, computational studies unravelled the conditions under which such networks display and sustain synchronized oscillatory activity. If the membranes of the interneurons depolarize (become activated) homogeneously and their synaptic connections hyperpolarize (become inhibitory) simultaneously, synchronized firing can occur at an oscillation period that is shorter than the duration of inhibition. In the case of hippocampal basket cells coupled through hyperpolarizing GABA_A synapses, such synchronization occurs in the γ frequency range.

Traub, Jefferys and Whittington's central hypothesis is that the homogeneous depolarization necessary for the interneuron network to oscillate is brought about by the action of a single class of neurotransmitter receptor — the metabotropic glutamate receptor. Despite this uni-causal viewpoint, which is by no means universally espoused, the beauty of this book arises from the fact that such hypotheses are clearly formulated, logically argued, then tested by experiments and computational modelling.

The activities of complex neural networks are not always based on complicated rules. Using pharmacological manipulations, the authors skilfully demonstrate the straightforward relationship between the frequency of the γ oscillation and the time course and amplitude of activity at GABA_A synapses. Furthermore, they use a two-site stimulation protocol in the CA1 region of a hippocampus slice as an *in vitro* model for long-range synchronization, and show how interneuron spike doublets are involved in this.

Overall, the book is succinct and informative, and leads the reader through difficult uncharted waters. Unfortunately, the data provided deal exclusively with the hippocampus, and it remains to be shown whether the authors' hypothesis holds true for the neocortex in general. Furthermore, the proposed role of metabotropic glutamate receptors in the generation of γ oscillations naturally invites a thorough review of the various subtypes of glutamate receptor to be found in the neuronal membrane. Finally, some parts of the book are heavy going. Facts are too often presented without being integrated into the authors' context and arguments.

Nevertheless, the interdisciplinary work summarized in the book is exactly the type of research necessary to fill the gap between mechanism and phenomenon in one of the fastest-moving fields in neuroscience. ■

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Diversity in the world of bugs

Ecology of Insects: Concepts and Applications

by Martin R. Speight, Mark D. Hunter and Allan D. Watt

Blackwell Science: 1999. 350 pp. \$55, £26.50

Alison F. Hunter

There is considerable disagreement over whether the total number of insect species is closer to 2 million or 10 million. What is agreed upon is that insects outnumber all other species combined. With an adaptable body plan, complex life histories and diverse feeding modes, insects have successfully radiated into every terrestrial nook and cranny. Ants forage in the desert at close to their lethal temperature of 55°C, apparently by accumulating heat-shock proteins before leaving cool tunnels. Some moths have a leisurely 13-year life cycle in the Arctic, growing only during the brief summer weeks.

Insects also engage in an extremely diverse range of ecological roles. Bark beetles consume wood by associating with cellulose-digesting fungi, while innumerable species consume more easily digestible insect tissues. Many insects pollinate plants, help decompose organic material and prey on pests.

Given this range, anyone writing a text on the ecology of insects requires considerable audacity to pull it off. Speight, Hunter and Watt provide solid coverage of current ideas. They discuss controversial issues ('talking trees' — the possibility that trees communicate about herbivory using volatile chemicals — overcompensation for herbivory and estimation of the number of insect species, among others) without getting bogged down by details of the debates, providing an even-handed, to-the-point treatment of issues.

It would be easy to write a text that would leave readers bewildered by masses of both ecological and entomological jargon. The authors, however, have taken great pains to explain terminology so that no great prior knowledge of entomology or ecology is required. The material is suitable for students of 18 years and up.

This is a fine volume with few defects. But the mosquito larva in Fig. 9.16 is upside-down, several of the figures are poorly labelled, and it is generally difficult to comprehend the figures from their captions alone. The specialities of the authors, on forest Lepidoptera and other economically important groups, are evident in the choices of examples, but the bias is not overwhelming. ■

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How many billions to go?

The peaking of the population growth rate deserves wider recognition.

Vaclav Smil

Some milestones in history are eagerly anticipated, much celebrated and repeatedly recalled: the first polio vaccine, the first landing on the Moon and the first non-stop transatlantic flight are good examples. Other milestones pass unnoticed and elicit surprisingly little comment, even when their importance eventually becomes obvious: global population milestones are among the best examples of this kind.

Some time in the first half of the seventeenth century, after millennia of stagnation or very slow annual growth amounting to small fractions of one per cent, the global population passed through the 'J-bend' of the exponential growth curve and began its still unfinished ascent. That this milestone went unnoticed is not surprising: that was the time when Galileo began observing the heavens with a telescope, when Europe was engulfed by a brutal religious war, when shoguns were consolidating their rule in Japan — and when nobody had any clear idea what the global count might be.

Curiously, modern society — obsessed with measuring everything, celebrating birthdays and giving exuberant welcomes to arbitrary dates ending in zeros — has passed two grand population milestones in a single generation, only acknowledged by a few experts. The first one was reached, virtually unnoticed, during the late 1960s, when the relative rate of global population growth peaked at just over two per cent a year. Slowly, but steadily, the rate kept falling (to about 1.7 per cent by 1990), but because of a growing base the absolute gains kept on increasing. Annual additions surpassed 80 million people during the late 1980s, and in 1991 the medium variant of the United Nations' (UN) forecast projected a gain of 100 million people a year by 1995 and more than 100 million people before the year 2000.

But, in the past few years, the UN demographers have had to cut their forecasts twice: fertilities have been falling more rapidly than anticipated, and the natural increase of the global population fell below 1.5 per cent during the early 1990s. As a result, the absolute increase peaked at 85 million people a year during the late 1980s and it was down to 80 million by 1995. The disintegration of the Soviet empire, and wars in the Gulf and the Balkans, overshadowed the second demographic milestone reached in a generation, as the absolute annual growth of humanity also began to decline.

What next? As with other species, the



Unnoticed milestone: the relative rate of world population growth peaked during the late 1960s.

exponential growth of humanity will end and the other bend of the growth curve will form, creating an S-shaped trace — but neither the onset of the S-bend nor the eventual maximum count can be predicted with great certainty. It is highly probable that we are already beyond the mid-point of the S-curve and that the current global total of six billion may not double again. The high variant of the UN's latest (1998) long-range forecast sees the global population growing to no more than 10.7 billion people by the year 2050. The low-growth scenario foresees just 7.3 billion people, and 8.9 billion might be the most likely outcome. Other projections, based on age structures, fertilities, mortalities and migration trends, support the UN's conclusion that we may not see another doubling of human numbers during the twenty-first century.

But the difference of more than three bil-

It is highly probable that we are already beyond the mid-point of the S-curve and that the global total of six billion may not double again.

lion people between the UN's low and high projections indicates that pinpointing the eventual value is beyond our ability, mainly because it is impossible to predict accurately the course of fertilities. Transition to low fertility has been accomplished in all affluent countries, the process is well advanced in most of Asia and Latin America, and there are clear indications that it is — finally — under way in sub-Saharan Africa, the largest remaining region of very high fertilities. The UN's medium (8.9 billion) forecast is based on the assumption that by the year 2050 total fertilities will be almost universally no higher than the replacement ratio of 2.1 children per woman, compared to the current global mean of about 2.7.

Will Ethiopia, with recent fertility close to seven, or Nigeria, Africa's most populous nation, with fertility near six, be able to change so fast? China has done so since the 1960s — but few would argue that its population controls could easily be copied in Africa. And so the world population may, after all, undergo one more doubling to 12 billion. Even so, we may be seeing the beginning of the end of the growth of our species. Children born today may be thinking about their retirement at a time when the global population count will have stabilized — or even begun to decline. □

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CORBIS/HENRY DITZ

Myosin steps backwards

Manfred Schliwa

All myosin motors move in the same direction — or so it was thought. The discovery of one form that acts in reverse sheds new light on the mechanics of these molecular motors.

Molecular motors are fascinating biological machines responsible for a variety of cellular movements. Some operate as individual molecules whose activities are visible only under sophisticated microscopes. Others cooperate in large ensembles of billions of molecules, allowing even gigantic animals to move. Yet the translocation of a tiny vesicle and the stomping of a dinosaur share an underlying mechanism at the level of a single motor molecule — the hydrolysis of one molecule of ATP leads to a small conformational change in the catalytic site. This change, in turn, is converted into a step of a few nanometres along a linear track.

One of the best-studied linear motors is a contractile protein called myosin. The myosins constitute a large superfamily subdivided into 15 classes according to their molecular structure and cellular function. Known myosins share one fundamental property — they all move in the same direction along an actin track. Actin is a protein that is found, among other places, in muscle fibres, and myosin moves towards the 'plus' end of the actin track (defined as the end at which subunits are added at a high rate). Now, on page 505 of this issue, Wells *et al.*¹ describe the stunning observation that a class VI myosin, which is thought to power movement of vesicles², tracks in the opposite direction — towards the minus end of the actin filament. The authors also offer a potential mechanism for this reversal of directionality.

Besides overturning the current dogma for myosin motors, this finding is significant in several respects. Based on structural studies of the class II myosins (which power the movement of all animals), force generation involves small conformational changes within the core of the motor molecule. These intramolecular movements communicate the hydrolysis of ATP to the base of a long, α -helical region — referred to as the lever — at the carboxy-terminal end of the myosin head³ (Fig. 1). The small conformational changes in the centre of the motor domain are thought to be translated into rotation of the lever, allowing its distal end to move through several nanometres. The subdomain at the base of the α -helix is pivotal (in the true sense of the word), because it anchors the point on which the lever rotates. This subdomain, which has been aptly

termed the converter⁴, helps turn the small conformational changes in the core of the motor domain into a much larger swing of the α -helix.

So, it was a logical move by Wells *et al.*¹ to start their search for a potential 'reverse' motor by looking for sequence peculiarities in the converter domains of the known myosin classes. It turns out that the class VI myosins possess a large (roughly 50-amino-acid) insertion at the transition from the motor core to the base of the α -helical lever, the converter domain. This insertion presumably allows the class VI myosin motors (and only this class) to move in the opposite direction to all other types of myosin.

How do small movements in the myosin core — which can be predicted to be similar for myosin motors of both directionalities — lead to a power stroke of the lever arm in opposite directions? A definitive answer will require structural information, at atomic resolution, of a class VI myosin head. In all likelihood, though, the mechanistic coupling in the converter region will differ between those motors that track towards the plus end of actin and those that move to the minus end.

Figure 2 shows a simplified mechanical model for how this might be accomplished. In this model, ATP hydrolysis induces small conformational changes, which are identical for both types of motor, in the core of the motor domain. These changes involve a

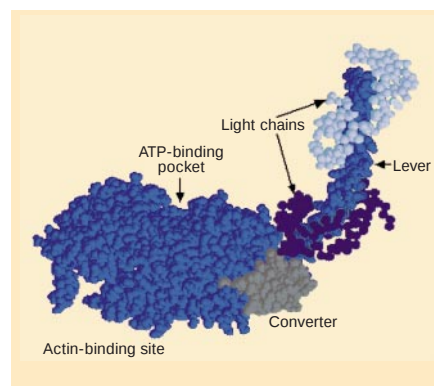


Figure 1 Space-filling model of myosin subfragment 1, indicating the position of the converter region (grey) at the base of the α -helical lever arm¹⁵. The positions of the ATP-binding site and the actin-binding site are indicated. Light chains are associated with, and stabilize, the lever.

helix termed the relay⁵, which links up the region near the ATP-binding pocket with the converter. So the relay communicates alterations in the status of ATP to the base of the lever. Minor rearrangements of the components in the converter region produce a swing of the lever in the opposite direction. Conceivably, the 50-amino-acid insertion in the converter region of myosin VI could accomplish that crucial change in structural connectivity.

The converter's ability to determine the polarity of movement invites a comparison with another family of molecular motors, the kinesins, members of which can move in one or the other direction along microtubules⁶. Atomic structures, in conjunction with functional studies, have given insights into the molecular basis of this directional bias⁷. Whereas the core structures of plus- and minus-end-directed kinesins are virtually identical^{8,9}, the topological relationships between the head and the adjacent neck domain differ considerably. There is strong evidence that the neck is crucial in determin-

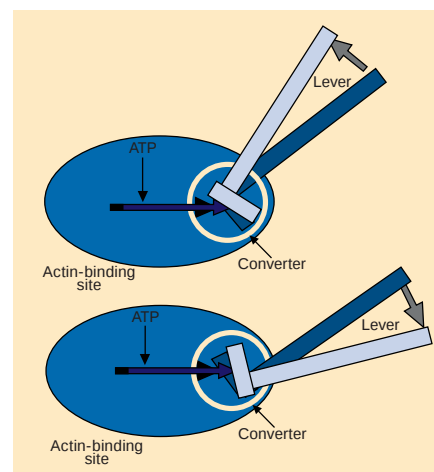


Figure 2 Simple mechanical model for myosin motors with opposite polarity of force generation. All myosins were thought to move in only one direction (towards the 'plus' end of actin), but Wells *et al.*¹ now show that class VI myosins move the opposite way, towards the 'minus' end. A slight difference in the topology of the rigid elements in myosin's converter region (white circle) means that the same small conformational change in the core of the motor domain (overlapping black and blue arrows) generates movement of the lever in opposite directions.

ing directionality^{10–13}. Thus, in both myosin and kinesin, the regions between the catalytic head and the adjacent stalk (the converter and neck, respectively) are important for specifying the direction of movement. And, although widely divergent in structure, these two regions seem to be functionally equivalent.

Now that a minus-end-directed myosin has arrived on the scene, and with the existence of minus-end-directed kinesins already well established, could the third class of linear motors, the dyneins, also have representatives that move in the other direction? All dyneins tested so far are minus-end-directed microtubule motors, but a provocative observation has come from studies of an exotic giant amoeba. In this cell, the kinetic and physiological properties of organelle movements along microtubules are characteristic of dynein going in both directions¹⁴.

So, we may have to watch out for a backward-stepping dynein as well.

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restricted by physical boundaries can a large thermal gradient arise. The boundary separating the silicate mantle from the iron-alloy core has a density contrast that is about twice that between the atmosphere and crust³. So any heat coming out of the core would form a thermal boundary layer at the bottom of the mantle. By estimating the concomitant temperature increase across the boundary, ΔT_{cm} , geophysicists would gain a valuable constraint on the total heat flux from the core, and insight into the core's physical and chemical properties.

Using geophysical, petrological and geochemical data, along with high-pressure experiments and geodynamic modelling, one can deduce that the temperature near the bottom of the mantle probably lies between 2,500 and 3,000 K (ref. 4). Estimates in the core rely on the assumption that the boundary between the solid inner core and the liquid outer core is at the melting temperature of the core material. Given that the core is made of iron with approximately 10% of lighter elements, and accounting for the fact that impurities tend to lower melting temperatures (although there is some evidence that oxygen in the core might increase melting temperature), a measurement of the melting temperature of iron at or near the pressure of the inner core boundary would probably place an upper bound on the core's temperature. Such estimates have been obtained by shock melting and by heating microscopic samples of iron in diamond-anvil high-pressure cells^{5–9}. The measurements are difficult and their interpretation is controversial¹⁰ — as shown in the 2,000 K

Earth science

Taking the core temperature

Mark S. T. Bukowinski

The Earth's core extends from its centre to about 55% of Earth's radius, or about 2,900 km below our feet. And yet its effects on humanity are immense. The liquid outer core generates the Earth's magnetic field that shields us, and all other living things, from the solar wind. And by some estimates it provides sufficient heat to the bottom of the mantle to influence mantle convection and hence tectonic motions, earthquakes and volcanism. The extent of these and other influences depends on just how hot the core is relative to the mantle.

This is a matter that continues to challenge geophysicists, and is the subject tackled by Alfè *et al.* on page 462 of this issue¹. The authors have applied new developments in computational physics to devise a virtual thermometer that may refine estimates of the temperature of the Earth's core. The technique involved is innovative and powerful, and should have much broader applications.

To appreciate the geophysical significance of Alfè and colleagues' contribution, consider first the temperatures in the Earth's mantle. Imagine going down a deep mine shaft. As you descend you start getting uncomfortably warm. Were you to go as deep as one kilometre, you would find that the temperature exceeds that at the surface by 20 to 30 °C. Vertical conduction along this temperature gradient is one mechanism by which the Earth rids itself of about 4.2×10^{13} joules of heat every second². How does the temperature trend continue with depth and how much of the surface heat flow comes from the core?

Were the temperature to continue rising at a rate of 20 to 30 °C per kilometre, rocks would start vaporizing at a depth of a few hundred kilometres. However in most of the mantle heat is transported by highly efficient thermal convection, which reduces the gradient by two orders of magnitude. Only where vertical convective velocities are

Box 1: *Ab initio* theory and the Earth

The chemical and physical properties of materials are described by quantum mechanics with great accuracy. However, for anything more complicated than the hydrogen atom, there are no exact solutions to Schrödinger's equation. The principal difficulty is the many-body nature of the problem, which is where density functional theory (DFT) comes in. This is based on the observation that the ground-state properties of materials are rigorously and uniquely determined by the one-electron density^{13,14}. The many-body Schrödinger's equation is replaced by an equation with effective interactions that depend only on the electron density. Although the exact form of these effective

interactions is not known, several approximations exist that yield very accurate structural and physical properties of molecules and condensed materials.

Ab initio DFT methods refer to calculations that rely on DFT but use no empirical information about the material. Given the atomic numbers of the constituent atoms, and known physical constants such as Planck's constant and electron charge, these calculations yield equilibrium structures, total energies, equations of state, vibrational frequencies and so on. Density functional theory has long appealed to geophysicists interested in understanding the physics of materials subjected to the extreme conditions in

deep planetary interiors. Not surprisingly, the Earth's core was the early subject of such investigations¹⁵. Today DFT methods are being used successfully to examine the effects of pressure on the structure, elasticity and thermodynamics of complex silicates thought to make up the deep Earth^{16,17}.

Developments such as that described by Alfè *et al.* promise that it won't be long before *ab initio* methods will be used almost routinely to study such geochemical processes as partitioning of elements among coexisting phases, high-temperature and high-pressure phase transformations, and even the chemical reactions between core and mantle materials.

M. S. T. B.

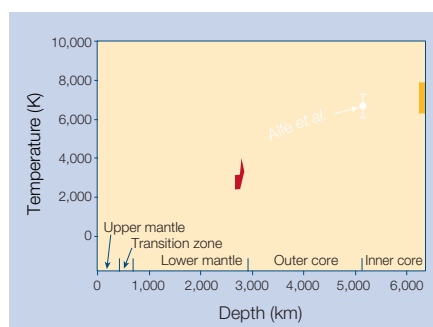


Figure 1 Estimates of temperature inside the Earth. This geotherm — geophysicists' best estimate of the radial distribution of temperature — is known only imprecisely. The lines provide rough estimates based on a variety of geophysical, geochemical and laboratory data, helped by geodynamic simulations. There are two major boundary layers at the top and bottom of the mantle. There may also be another sharp increase in temperature at 660 km, the boundary between the lower mantle and the transition zone, if this boundary separates layers of distinct chemical composition. The geotherm is highly uncertain at the core–mantle boundary and within the core. Bounds within the core are based on the range of measured melting temperatures of iron. The circle with an error bar shows the estimated melting temperature of iron at the inner core boundary as calculated by Alfè *et al.*¹.

span of temperatures that can be deduced from these experiments (Fig. 1).

Now a new player joins the quest. Alfè *et al.* use an *ab initio* molecular dynamics method¹¹ (Box 1), combined with a novel 'thermodynamic integration' scheme, to compute the free energies of solid and liquid iron as functions of pressure and temperature. By comparing these energies, they arrive at a melting curve that, the authors claim, competes in accuracy with existing experimental data. The calculated melting curve, which gives a temperature of $6,670 \pm 600$ K at the inner core boundary, is consistent with a calculation based on shock-wave data⁵ and the diamond-anvil measurements of Williams *et al.*⁶.

Alfè and colleagues' prediction leaves much uncertainty in the value of ΔT_{cm} . But the real significance of their contribution rests in its being the first completely *ab initio* calculation of the thermal properties of solid and liquid iron, and hence its melting curve, and the fact that its accuracy seems to be well supported by previous calculations of thermodynamic properties of solid and liquid iron. Other computations, for example those of Stixrude *et al.*¹², amply demonstrate the utility and credibility of *ab initio* theory. Nevertheless, only the future will tell whether the calculated iron melting curve is indeed accurate, just as it will tell which, if any, of the measured melting curves are accurate.

Meanwhile, this new study by Alfè *et al.* provides a benchmark for future computations and challenges the experimental community to try to converge on tighter limits on the melting temperatures. Further stimulus will probably come from computations of chemical potentials that should yield theoretical estimates of the solubility and thermal effects of sulphur, oxygen, hydrogen and other candidate alloying elements in the core. Such calculations should also allow estimates of the density change across the inner core boundary, as well as of the heat of fusion.

By combining *ab initio* computations with geophysical data it will then be possible to test the assumptions underlying the gravitational dynamo models: that the light component of the core is largely excluded from the inner core, leaving behind a buoyant residue that can drive convection in the outer core. The Earth can be thought of as a high-pressure experiment, a vast arena for the interplay of geophysical observation with experimental and computational materials science. For research, it is a clear win–win situation. ■

Apoptosis

Cutting red-cell production

Stuart H. Orkin and Mitchell J. Weiss

For the proper delivery of oxygen to our tissues we need to have enough circulating red blood cells (erythrocytes). As Olympic officials have learned, artificially boosting the number of erythrocytes enhances athletic performance. But a potential downside of having excessive red blood cells is sluggish circulation and stroke. So, for good health, we need finely tuned regulation of red-cell production, and on page 489 of this issue DeMaria and colleagues¹ propose a new mechanism for how this might be accomplished.

The maturation of immature red-blood-cell precursors (proerythroblasts) into mature erythrocytes is positively controlled by a polypeptide hormone called erythropoietin, which promotes both proliferation and survival of erythroid precursors. The idea that the formation of red blood cells might also be negatively regulated has only recently been considered. In cell culture, binding of ligands to so-called death receptors on the surface of erythroid precursors activates death-promoting enzymes called caspases (cysteine proteases with aspartate specificity), and culminates in cell suicide (apoptosis). DeMaria and colleagues¹ now provide evidence that activation of death receptors on erythroid cells — or, alternatively, deprivation of erythropoietin — leads to caspase-induced cleavage (and, hence,

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inactivation) of a nuclear regulatory protein called GATA-1. This transcription factor is crucial for the maturation and survival of erythroid precursors. The authors' observations suggest a hitherto unknown negative-control mechanism by which life, death and cellular maturation decisions converge on a single protein target (Fig. 1, overleaf).

In mammals, red blood cells form in the bone marrow within anatomical units called erythroblastic islands. These units comprise macrophages (white blood cells that engulf foreign particles and microorganisms) surrounded by erythroblasts at different stages of maturation. The proliferation and survival of these erythroblasts depend on the presence of erythropoietin. Erythroblasts have erythropoietin receptors on their surface and, if deprived of erythropoietin, they succumb to apoptosis. In part, this reflects a requirement for signals from the erythropoietin receptor in inducing or stabilizing the expression of an anti-apoptotic protein called bcl-x_L (ref. 2). Indeed, as shown by a study of embryonic stem cells³, bcl-x_L is also necessary for normal maturation of erythroid cells. So, as with many other types of cell, apoptosis must be held in check for normal development to ensue.

As well as extracellular anti-apoptotic signals, erythroblasts use internal programmes to ensure their own survival. The

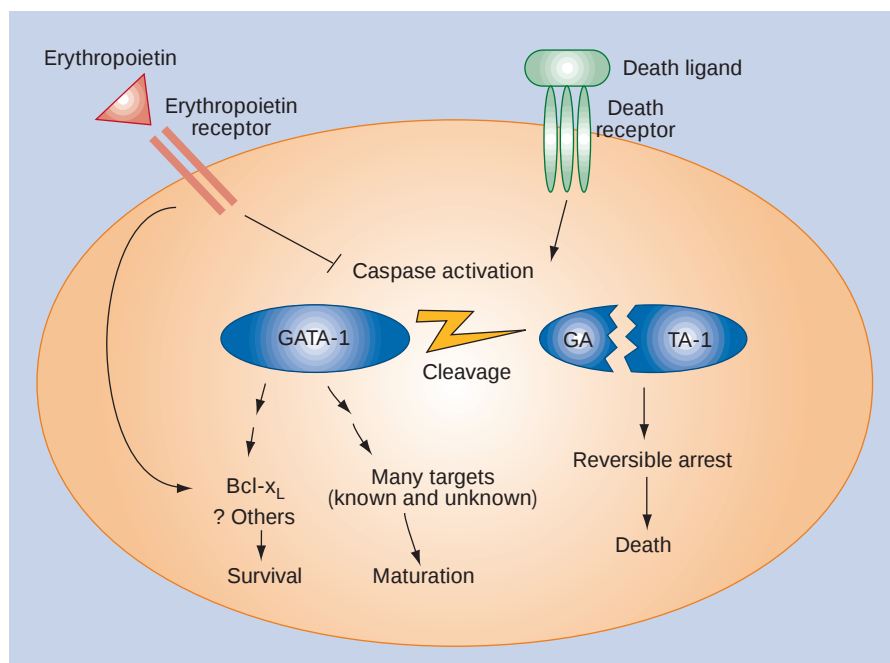


Figure 1 Convergence of opposing pathways on the transcription factor GATA-1 to regulate the survival and maturation of red blood cells. As proposed by DeMaria *et al.*¹, signalling through death receptors leads to activation of caspases, which cleave (thereby inactivating) GATA-1. The survival, maturation and cell-death programmes are impaired differentially, presumably depending on the residual level of native GATA-1 and on whether or not the erythropoietin receptor has been activated by its ligand erythropoietin. Signalling through the erythropoietin receptor promotes cell survival, partly through activation of bcl-x_L expression, partly by blocking caspase activation. The precise mechanisms remain to be worked out.

transcription factor GATA-1, which is abundant in erythroid precursors, is essential for maturation of erythroblasts⁴ — the absence of GATA-1 leads to apoptosis and a block in maturation^{5,6}. Not only is its presence necessary, but the levels of GATA-1 influence cellular maturation⁷. Some of the target genes regulated by GATA-1 are likely to be intrinsic to cell survival, although their identities are largely unknown. However, GATA-1 strongly induces expression of bcl-x_L, and it also cooperates with erythropoietin signalling in this regard³. So, the erythropoietin and GATA-1 signalling pathways converge on at least one target of the cell-survival programme.

Although death receptors are used by many types of cell⁸, their potential functions in erythroid cells are only now coming under scrutiny^{9,10}. Under conditions where levels of erythropoietin are limiting, apoptosis of erythroid precursors is induced by activation of a death receptor called Fas by its ligand (the aptly named Fas ligand; FasL). DeMaria and colleagues have previously shown¹⁰ that Fas is expressed at most stages throughout the maturation of human erythroid cell, whereas FasL is expressed only by more mature erythroblasts. This observation allows for a model in which mature erythroblasts participate in a negative-feedback loop to inhibit the formation of red blood cells by activating the Fas/FasL system in immature erythroblasts. In this way, the levels of red blood cells

being produced might be tightly regulated.

In pursuing this line of reasoning, DeMaria *et al.* have now¹ examined the consequences on erythroid-cell maturation of activating death receptors in a highly purified human cell-culture system. A variety of death receptors and caspases are expressed in erythroid cells. Using anti-Fas antibodies at a concentration that does not induce apoptosis in the presence of erythropoietin, the authors found that maturation of erythroid cells is, indeed, blocked by activation of the Fas/FasL system. This effect could be reversed if the cells were treated with a caspase inhibitor.

DeMaria and colleagues observed a similar inhibition of maturation in a related blood lineage (megakaryocytes). Again, this effect was prevented by treatment with a caspase inhibitor. The authors also found that, remarkably, treatment of erythroid cells with anti-Fas antibodies leads to selective cleavage of GATA-1. Critical to interpretation of the experiments was the fact that when the authors introduced a modified form of GATA-1, which is resistant to caspase-mediated cleavage, maturation of the anti-FasL-treated cells was restored. Moreover, expression of caspase-resistant GATA-1 allowed for considerable cell survival and maturation in the absence of erythropoietin. And, consistent with this finding, expression of a dominant-negative form of caspase-8 abolished the dependency on erythropoietin. So, sig-



100 YEARS AGO

The surface of the globe has not always been as we now see it. When, in the past, the surface had a temperature of about 400 °F., what is now the water of the ocean must have existed as water vapour in the atmosphere, which would thereby — as well as because of the presence of other substances — be increased in density and volume. Life, as we know it, could not then exist. Again, science foresees a time when low temperatures, like those produced by Prof. Dewar at the Royal Institution, will prevail over the face of the earth. The hydrosphere and atmosphere will then have disappeared within the rocky crust, or the waters of the ocean will have become solid rock, and over their surface will roll an ocean of liquid air about forty feet in depth. Life, as we know it, unless it undergoes suitable secular modifications, will be extinct. Somewhere between these two indefinite points of time in the evolution of our planet it is our privilege to live, to investigate, and to speculate concerning the antecedent and future conditions of things.

From *Nature* 28 September 1899

50 YEARS AGO

Except for the input and output mechanism, the EDSAC [electronic delay storage automatic calculator] has no moving parts, all computing and control operations being performed by means of electronic circuits. Within the machine numbers are expressed in the scale of two and are represented by trains of pulses synchronized with a continuously running 'clock pulse' generator. ... Numbers expressed in this form are stored by a method depending on the use of an ultrasonic delay unit. The pulses are applied to a quartz crystal mounted at one end of a column of mercury, and give rise to ultrasonic pulses which travel through the mercury with the velocity of sound. On arrival at the far end they strike a second quartz crystal and are reconverted into electrical pulses. The time taken to traverse a column of mercury 5ft. long is about 1 msec., and the interval between the beginning of one pulse and the beginning of the next is 2 μsec.; there can thus be as many as 500 pulses passing down the column at any one time. On emerging from the delay unit the pulses are amplified and reshaped, and passed back to the input of the delay unit. They then continue to circulate indefinitely and are available when required.

From *Nature* 1 October 1949.

nalling through the erythropoietin receptor and activation of death receptors seem to compete through GATA-1 to elicit one of several possible cell fates — differentiation, reversible maturation arrest or death.

The findings of DeMaria and colleagues emphasize the close relationship between cell-survival and cell-maturation programmes, as well as the intricate balance between positive and negative influences in regulating cellular processes (Fig. 1). We know that external cues — such as exposure to FasL or erythropoietin — impinge on the internal programmes controlled by GATA-1 to modulate cellular maturation. But questions remain. First, what is the physiological role of the negative control of red-blood-cell formation through death receptors? To date, observations have been limited to culture systems. Nonetheless, the increased levels of soluble FasL found in people with anaemia, and the excessive activation of death-receptor pathways in chronic disease, suggest potential biological relevance¹¹.

Second, the proposed main site at which caspases cleave human GATA-1 is not conserved in the mouse sequence. Does this indicate that other sites are used as well, or that there are species-specific differences? Third, does caspase-mediated cleavage target critical nuclear regulatory proteins in

other types of differentiated cell? For example, might GATA-3 be a substrate in T cells? Caspase-induced death has been likened¹² to a “well planned and executed military operation”. In this context, using the inactivation of GATA-1 by caspases to negatively regulate the formation of red blood cells is analogous to using a laser-guided smart bomb — a tactical strike at the command centre of the differentiation pathway.

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component dioxygen, O₂) as the oxidant, and non-toxic solvents such as water or supercritical carbon dioxide. Unfortunately, air oxidations are intrinsically non-selective and difficult to control². Such chain reactions involving free radicals are either too slow (for example, ageing of human tissue, plastics or metal surfaces) or too fast (for example, combustion) to be useful, except in fuel technology. In addition, the metals in homogeneous catalysts tend to precipitate from water as catalytically inactive and insoluble metal oxides.

On the biological front, ADHOC-99 offered insight into the mode of action of both the soluble³ and membrane-bound⁴ forms of methane monooxygenase (sMMO and pMMO respectively). MMO catalyses the dioxygen oxidation of methane — the most abundant hydrocarbon and the principal constituent of natural gas — to methanol, a process of both fundamental and practical interest. The underlying attraction lies in the challenge of selectively cleaving the robust C–H bonds of methane and accumulating high enough yields of methanol product, because methanol is much more reactive in air than methane itself. From a practical outlook, methanol has almost the same energy content as methane, but unlike methane it is a relatively safe liquid that is inexpensive to transport and store. It was established that the well studied Fe-containing sMMO (H. Dalton, Univ. Warwick) and the less understood Cu-containing pMMO (S. Chen, Caltech, Pasadena and Academia Sinica, Taipei) share many features. Both exert their remarkable selectivities in part by restricting the size of molecules that can reach the metal-containing active sites. In both cases shape-specific hydrophobic sites further increase selectivity by favouring binding of methane over the methanol product. Complex interactions between multiple protein components of both sMMO and pMMO modulate the flow of electrons to the metal-containing active sites and regulate overall reactivity.

Biological enzymes can also be more effective (that is, greener and more selective in chemical synthesis) than traditional inorganic reagents. Oxidases or microorganisms that contain these enzymes have been used to selectively produce a variety of chiral organic products of commercial value to pharmaceutical and other industries, such as chiral alcohols (W. Adam, Univ. Würzburg). Such biocatalytic oxidations will play a growing role in the greening of technologies that are dependent on this chemistry.

On the synthetic front, lessons learned from studies of oxidation enzymes, as well as structure–activity information from oxidations catalysed by soluble metal complexes have contributed to the production of promising new oxidation catalysts. One group managed to immobilize (on modified

Homogeneous catalysis

Controlled green oxidation

Craig L. Hill

The ability to selectively transform organic and biological materials by removing electrons and/or adding oxygen atoms (oxidation) is pervasive in biology and critical to the pharmaceutical, petrochemical, agricultural and other industries. Unfortunately, the catalysts available today for homogeneous oxidation (reactions in which both catalyst and reactants are in the same phase) are generally far from ideal. The oxidation catalysts of the future will need to be highly selective, environmentally benign and highly stable (Fig. 1). Chemists and biochemists recently gathered at the ADHOC-99 meeting to report progress in understanding these factors and in developing new homogeneous oxidation catalysts*. Investigations into exquisitely selective biological catalysts, such as the oxidase and oxygenase enzymes, and many metal-containing synthetic catalysts are guiding us towards the ideal oxidation catalyst.

Selectivity in catalytic oxidations, as with other chemical transformations, is crucial.

Generating the desired product in low yield wastes money and natural resources and low selectivity means toxic or otherwise unacceptable by-products¹. In general, catalyst stability often dictates economic viability. ‘Green’ oxidation requires the use of hydrogen peroxide or ideally air (or its reactive

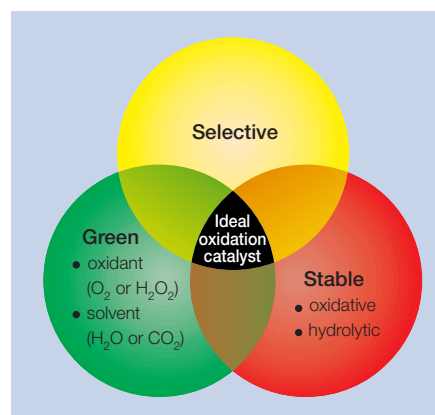


Figure 1 The ideal homogeneous oxidation catalyst. The oxidation catalysts of the future will have to display all three of these inter-related attributes.

*Seventh International Symposium on Dioxygen Activation and Homogeneous Catalytic Oxidation (ADHOC-99), York, UK, 19–23 July 1999.

silica) soluble manganese complexes used for peroxide bleaching⁵ so that they are recovered more easily after the reaction (D. De Vos, Katholieke Univ. Leuven). They also reported effective new homogeneous oxidations, including olefin epoxidations, catalysed by these manganese complexes. Another group synthesized titanium-containing silicate cage molecules that represent structural and functional analogues of TS-1, the titanium-containing zeolite that has become one of the most commercially significant catalysts for green peroxide oxidation of organic molecules⁶. Reactions of these soluble cage compounds with peroxides and alkenes contribute to the growing evidence that the active sites in TS-1 involve a tripodal centre, (SiO)₃Ti(OH), in which the titanium is bound by three oxygen atoms of the microporous solid (M. Crocker, Shell Res., Amsterdam). Also reported at the meeting was a successful air-based oxidation that was a green version of the Wacker reaction — one of the largest homogeneous catalytic processes involving dioxygen oxidation of ethylene to acetaldehyde⁷. In this reaction, chelating diamine–palladium complexes catalyse the oxidation of olefins to ketones without using two undesirable ingredients currently

required by the Wacker process — copper and chloride (R. Sheldon, Delft Univ. Tech.).

Significant steps have been made in understanding and controlling selectivity in homogeneous oxidation reactions and in producing high-value oxidized organic materials with minimal environmental cost. But catalyst stability is generally ignored and it directly affects both selectivity and green operation (Fig. 1). If a catalyst is damaged during operation (turnover) it often transforms to a species that remains active (consuming substrate and resources) but one that now generates useless or harmful products. In this way catalyst damage can undo carefully crafted gains in other areas. All three intersecting requirements must be considered in the future to produce the ideal homogeneous oxidation catalyst. ■

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Physiology

The haemoglobin enzyme

Kiyohiro Imai

Haemoglobin is well known for its function in the vascular system of animals, transporting oxygen from the lungs or gills to peripheral tissues. It also aids, both directly and indirectly, the transport of carbon dioxide and regulates the pH of blood. Three years ago, a fourth function for mammalian haemoglobin was proposed¹ by J. S. Stamler and his collaborators — they suggested that the allosteric mechanism of haemoglobin is involved in the nitric oxide (NO)-mediated regulation of blood flow. Haemoglobin is a model allosteric protein², yet it has no catalytic activity, leading the late Jeffries Wyman (a prominent protein scientist) to dub it an “honorary enzyme”^{2,3}. Now, a report by Minning *et al.*⁴ on page 497 of this issue effectively removes that honorific by showing that haemoglobin from a parasitic nematode, *Ascaris suum*, is a true enzyme, serving as an NO-activated deoxygenase.

By its original definition, the term haemoglobin is used for the proteins contained in blood, haemolymph or coelomic fluid. However, proteins in other, non-circulating fluids or in single-celled organisms may also have this name. Haemoglobin is widely distributed among prokaryotes, unicellular eukaryotes, plants and animals. It

comprises a polypeptide chain called globin, associated with protohaem IX — a planar complex of iron and protoporphyrin IX. These structural units form tetramers in vertebrate red blood cells, but in invertebrates the combinations are more diverse, ranging from the monomeric haemoglobin of protozoa to the gigantic annelid extracellular haemoglobin which has 144 subunits. The physiological function of haemoglobin is attributed to its ability to bind dioxygen reversibly, depending on the partial pressure of oxygen (PO_2). To function as an oxygen carrier, the oxygen affinity of haemoglobin (which is measured by the reciprocal of PO_2 at 50% saturation) must be comparable to PO_2 in the environment.

The haemoglobin found in the peritenteric fluid of *Ascaris* worms has one of the highest observed affinities for oxygen. However, this tight binding makes *Ascaris* haemoglobin unsuitable as an oxygen carrier. So what is its physiological function? Based on biochemical experiments, Minning *et al.*⁴ now propose that *Ascaris* haemoglobin is an NO-dependent ‘deoxygenase’, which uses endogenously produced NO as a substrate to detoxify oxygen. They have come up with a model that includes more than ten steps, proceeding through an inter-

play of the haem iron and several amino acids of the protein moiety (the haem pocket; see Box 1, overleaf).

The physiological role of *Ascaris* haemoglobin was previously proposed to be a ferrihaemoprotein reductase activity in the squalene epoxidation reaction, a pathway for sterol biosynthesis⁵. But this is now in doubt because sterols are essential nutrients, so it is unlikely that they are synthesized *de novo* in *Ascaris*⁶. Instead, Minning and colleagues suggest that, in contrast to most other haemoglobins, *Ascaris* haemoglobin’s physiological function is not to supply oxygen but to eliminate it. The deoxygenase activity removes oxygen toxicity by keeping tissues highly anaerobic. This makes sense because *Ascaris* worms inhabit the anaerobic intestines of their host and have a fully anaerobic mitochondrial oxidation pathway that is poisoned by even trace amounts of oxygen⁶. A similar role as an oxygen scavenger has been proposed⁷ for leghaemoglobin, which is found in the root nodules of legumes. This haemoglobin has a very high affinity for oxygen, keeping the symbiotic nitrogen-fixing bacterium found in the root nodules anaerobic and protecting the nitrogen-fixation enzyme system from oxidation. However, unlike *Ascaris* haemoglobin, leghaemoglobin is not a sink of oxygen — that is, the oxygen is trapped but not metabolized.

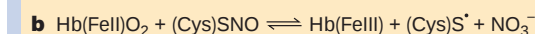
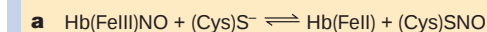
The globin–haem complex can be adapted to various biological functions through the design of the haem pocket. By replacing only a few amino-acid residues, the oxygen affinity can be varied up to 27,000-fold, and the reversible oxygen-binding ability is even replaced by catalytic activities involving O_2 metabolism. Take the report by Lebioda *et al.*⁸ on page 445 of this issue. These authors have found that a dehaloperoxidase isolated from *Amphitrite ornata*, a terebellid polychaete, evolved from a globin. It still has the three-dimensional ‘globin fold’ (a characteristic of haemoglobin), and it retains its ability to bind oxygen. But whereas this enzyme evolved from a globin, the recently discovered indoleamine dioxygenase-like myoglobins are haemoproteins that have attained the ability to bind oxygen reversibly through convergent evolution from indoleamine dioxygenase⁹.

Despite a tremendous number of studies, all the mysteries of haemoglobin have not yet been solved. Future work will extend our knowledge in two main directions. The first involves haemoglobin’s reactions with NO, such as its S-nitrosylation action (explored by Stamler’s group)^{1,4} and its function as a negative allosteric effector by ligating haem, as studied by Yonetani’s group¹⁰. The second is likely to encompass the origin and evolution of globin, including the functional diversity of the haemoglobins in various organisms. ■

Box 1: How does haemoglobin eliminate oxygen?

The endogenous NO is bound to, and oxidizes, the haem iron, which is in equilibrium with S-nitrosothiol ('thiol') being an organic compound with an -SH group) of a cysteine residue (cysteine E15) in the haem pocket. The equation for this reaction is shown in **a**. Oxygen is bound to the ferrous (FeII) ion, forming an unstable peroxynitrosyl complex with the S-nitrosothiol, which then decomposes to generate nitrate (**b**), where $\cdot$ of (Cys)S represents an unpaired electron.

For these reactions to occur, the system requires an electron mediator, NADPH, which reduces the thiyl radical produced in reaction **b**. This NADPH-dependent, NO-activated deoxygenase activity of *Ascaris* haemoglobin depends on the unique structure of the distal side of



the haem pocket. Glutamine E7 and tyrosine B10 — which are replaced by histidine and leucine, respectively, in normal haemoglobins — form strong hydrogen bonds with the bound dioxygen, forming the basis of the *Ascaris* haemoglobin's extremely high affinity for oxygen. Cysteine E15 lies in close proximity to the oxygen-binding site. So, the distal haem pocket of *Ascaris* haemoglobin is designed to facilitate the interaction between the haem-bound oxygen and NO, which is bound to the -SH group of cysteine E15.

Minning *et al.*⁴ point out that the position of this

cysteine residue is important in determining how NO is used. If the cysteine is located on the distal side of the haem pocket, it allows the NO-mediated enzymatic consumption of O₂. But if it is located on the proximal side (take, for example, cysteine F9 of mammalian haemoglobin), it allows the NO-mediated control of O₂ delivery. Incidentally, bacterial flavohaemoglobins detoxify NO enzymatically but do not use thiol. So, from the viewpoint of NO, the nematode haemoglobin is an evolutionary bridge between the primordial flavohaemoglobin and the mammalian haemoglobin. **K. I.**

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Planetary systems

Ageing dust fades away

Sergio Fajardo-Acosta

Fifteen years ago, astronomers discovered that many nearby stars are surrounded by solid material such as dust. Ever since, we have wondered whether this dust resembles the disk of raw material that gave birth to our own planetary system 4.5 billion years ago. On page 456 of this issue, Habing *et al.*¹ report infrared observations of dust around stars of various ages. They discover that this dust disappears 300–400 million years after the birth of the stars, similar to what is believed to have happened to the dust disk in our Solar System when planets formed.

To understand the formation of our Solar System, and in particular the evolution of its solid material — we happen to be standing on a big chunk of it — it is useful to look at nearby stars that could also have solid mater-

ial around them. Looking at these other systems is like taking pictures in the middle of a forest to figure out the life history of a single tree. By observing multitudes of stars at various stages, astronomers have pieced together the path of stellar evolution. The Hubble Space Telescope image of an ionized hydrogen region in the Eagle Nebula (M16) provides a spectacular glimpse of stellar evolution in action², showing stars being born out of their cocoons. Although it is observationally more challenging, we are now starting to follow the evolution of the solid material around these stars as it develops into planetary systems.

In 1984, astronomers set out to make routine observations of bright main-sequence stars — those in their longest and most stable evolutionary stage — with the

Infrared Astronomical Satellite (IRAS). To their surprise they discovered that the star α Lyrae (also known as Vega) emitted almost ten times more infrared radiation than expected from a normal main-sequence star³. This finding was the tell-tale signature of a cloud of infrared-emitting dust around α Lyrae. Similar discoveries followed, mainly with IRAS, and to date more than 100 main-sequence stars are known to harbour so-called Vega-type dust around them, presumably in disk structures.

A puzzling aspect of these Vega-type dust disks is their very existence today. Dust grains do not keep on orbiting a star forever. They are destroyed by collisions with other grains or by sublimation from stellar heating; some grains are pushed away from the system by radiation pressure; other grains spiral into the star due to the opposite effect, by which stellar radiation causes drag. The gravitational perturbations from large bodies (such as planetesimals and planets) can also eject dust out of the system, or inject it close to or into the star. The result is that Vega-type dust grains survive for only a fraction of a main-sequence star's lifetime. The inescapable conclusion is that the Vega-type dust that we see must be continuously replenished^{3,4}.

Vega-type dust can be replenished by the grinding together of larger bodies, such as fledgling planetesimals, comets or perhaps even failed planets. Later on, fully formed massive planets will gravitationally clear up most of the Vega-type dust disk over long timescales. Studies of a handful of Vega-type systems suggest that these disks do thin-out (become less dusty) with age^{5,6}. The new study by Habing *et al.*¹ is significant because of the large number of stars (84) for which they determined approximate ages and whether they had infrared-emitting dust around them. These observations were carried out with the Infrared Space Observatory (ISO), with the aim of improving on previous, less sensitive IRAS observations.

Determining the precise ages of stars remains a tricky problem. One needs to know fairly well their temperatures, intrinsic luminosities and metallicities (the fraction of heavy elements they contain), to fit theoretical stellar evolution models — themselves not iron-clad. Even the famous star β Pictoris, whose dust disk was the first to be imaged⁷, still has an uncertain age estimate (a few 10 to 100 million years⁸). The ages determined by Habing *et al.*¹ have in some cases uncertainties of around 50%.

Despite these uncertainties, Habing *et al.* found from their sample that about 60% of stars younger than 400 million years have dust disks, whereas only about 9% of those older than that have them. Apparently, 90% of stars with dust disks lose them after 300 to 400 million years. Were there parallel events in the history of our Solar System, at those

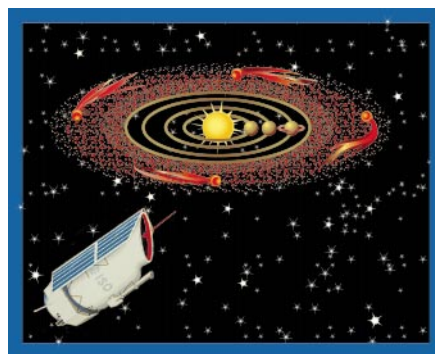


Figure 1 The Kuiper Belt of our Solar System (shown in red) harbours solid material (such as comets) in a disk extending beyond the orbit of Pluto. The Kuiper Belt is believed to have been much dustier in the past, when planets were forming. Other nearby stars, younger than the Sun, also have possible 'Kuiper Belts', as dense as ours was 4.5 billion years ago. These dust regions are detectable in infrared radiation, best studied with space telescopes such as the Infrared Space Observatory (ISO). New observations of nearby stars by Habing *et al.*¹ suggest that these regions thin-out as the stars age, possibly because of interactions with newly formed planets around them.

ages, that could tell us why these disks disappear? The answer is yes, if we first recognize that the Vega-type disks seen by IRAS and ISO are similar to the Kuiper Belt of our Solar System — the disk-like reservoir of short-period comets, extending beyond the orbit of Pluto (Fig. 1). The only difference is that our Kuiper Belt contains roughly 100–1,000 times less dust than typical Vega-type disks (before they disappear).

Habing *et al.* note that models of our Kuiper Belt show that it underwent 'erosion' because of gravitational perturbations from the newly formed giant planets. Around that time, about a hundred comets per year were thrown from the Kuiper Belt into orbits so close to the Sun that they almost certainly vaporized. At present there are only a couple of such Sun-grazing comets per year. These processes probably led to the thinning out of our Kuiper Belt to its current low density of dust. Tantalizingly, these events took place 400–800 million years after the birth of the Solar System, strongly suggesting that we are now witnessing the same processes in the 'disappearing' Vega-type disks.

Vega-type disks are attractive laboratories in which to study the effects of planets on the disks out of which they form. There is already one case of a star where both a planet and a Vega-type disk have been observed: ρ^1 Cancri (refs 9,10). This system is open to observations at high spatial resolution to see the effects of a known planet on its 'Kuiper Belt' disk. More of these systems need to be found. The work of Habing *et al.*¹ can be extended to quantify the amount of dust (rather than just noting its presence or

absence) in many systems, and seeing how this amount decreases with age. Detailed infrared observations will let us more fully understand what planets do to their disks — or to whatever is left of them!

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Cell biology

A cell for all reasons

William F. Loomis and Robert H. Insall

Nature has come up with a tremendous variety of organisms with diverse shapes, sizes and life styles that nonetheless all work on a common framework of cellular mechanics. To understand these shared mechanics, few experimental organisms offer the versatility of the soil amoeba *Dictyostelium discoideum*. Growing cells of this eukaryote make a living as asexual unicellular phagocytes, voraciously engulfing bacteria or yeast, and they can double their numbers in as little as four hours. When starved, they perform an about-face, aggregate into groups of up to a half a million cells, and develop as multicellular organisms.

Scientists who work with *Dictyostelium* met* last month to discuss the latest research on topics as diverse as signal transduction, macropinocytosis, centrosome separation, and the involvement of factors such as STATs, NF- κ B and Myb in gene transcription. A *Dictyostelium* Genome Project is under way (see Box 1), and the ability to recover genes electronically rather than by

laborious probing of clone libraries has already revolutionized the way *Dictyostelium* is studied.

Starving cells aggregate using chemotactic mechanisms that are apparently identical to those seen in leukocytes^{1,2}. *Dictyostelium*'s advantages in experimental accessibility have stimulated intense interest in the molecular details, and green fluorescent protein (GFP) has been fused with various components involved in chemotactic cell movement (Fig. 1; P. Devreotes, Johns Hopkins Univ.; R. Firtel, Univ. California San Diego). In this way the seven-transmembrane receptor, CAR1, has been shown to be evenly distributed over the cell surface, and to remain so when the receptor is stimulated. On the other hand, fusions of GFP with the β -subunit of heterotrimeric G-proteins appear as a shallow anterior–posterior gradient.

More exciting still, several proteins that carry lipid-binding pleckstrin-homology (PH) domains are rapidly recruited to the plasma membrane at the front of cells undergoing chemotaxis. These include

Box 1: The *Dictyostelium* genome project

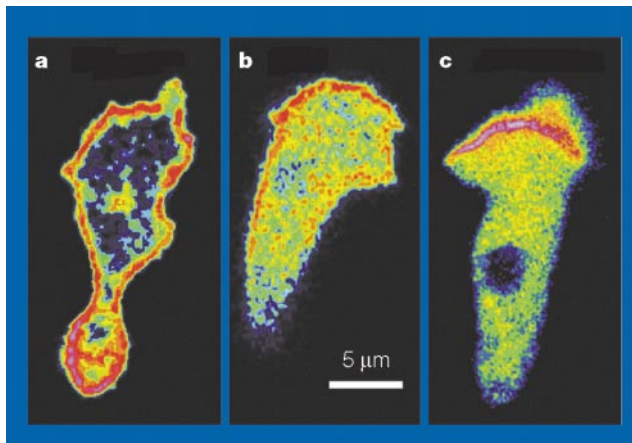
Sequencing of the six-chromosome *Dictyostelium* genome, a project involving groups in Cambridge, UK, Houston and Jena, began in 1998 and has a projected date of completion by 2002. The strategy being followed is a modified whole-genome shotgun approach, coupled to compilation of a high-resolution (± 5 -kilobase) physical map. Over two-fold coverage of the 34-megabase genome is now available and there is already information on at least 90% of

the genes. Assembly of contiguous sequences will initially centre on chromosomes 2 and 6, with the others following in the next few years. In addition, the first stage of a large-scale project analysing complementary DNA has been completed in Japan and the sequences of several thousand genes expressed during development are available online⁶. Proteomic studies and massively parallel gene expression analyses using DNA microarrays are now underway

to elucidate the function of many of the newly uncovered genes. As networks are recognized they can be rapidly tested using the techniques of molecular genetics that are so powerful in this haploid organism. Genes can be overexpressed in either pre-stalk or pre-spore cells and can be knocked out by homologous recombination. Development of genetic mosaics, epistasis and suppressor studies can establish order in signalling pathways. **W.F.L. & R.H.I.**

Figure 1 Localization of signalling events in *Dictyostelium* cells undergoing chemotaxis. Each cell was stimulated by chemoattractant (top of each frame).

Chemoattractant receptors (a) and G-protein β -subunits (b) are uniformly distributed on the cell surface, whereas proteins with lipid-binding pleckstrin-homology domains (c) are selectively recruited to the leading edge. The distribution of each protein was imaged in cells where the endogenous gene was replaced by a green-fluorescent-protein fusion construct. Regions of highest fluorescent intensity are in red.



CRAC, a protein necessary for receptor activation of adenyl cyclase; the protein kinase, PKB, which is required for cell polarization; and PHD1, which is required for chemotaxis. Membrane association of the PH-domain proteins can be blocked by inhibitors of phosphatidylinositol-3-OH kinase (PI(3)K) or null mutations in two partially redundant PI(3)Ks. These results show that cellular polarity can now be analysed at the molecular level.

Green fluorescent protein has also been used to follow the behaviour of differentiated pre-spore and pre-stalk cells as they sort from each other to form specific tissues^{3,4} (J. McNally, Natl Insts Health; H. Levine, Univ. California San Diego). Videomicroscopy and computer simulations show that changes in relative adhesion may be important in cell sorting. Expression of two proteins connected with cell adhesion, lagC (a relative of I-CAM) (C.-H. Siu, Banting and Best Inst., Toronto) and ampA (which is related to the disintegrin family) (D. Blumberg, Univ. Maryland), is restricted to the stalk-cell precursors. The phenotypes of null mutants in either gene are completely consistent with roles in cell-type-specific adhesion. The GFP approach has also been used to follow the way individual cells move within the multicellular organism as it responds to light (K. Miura, Univ. Munich) or to waves propagating from the anterior (C. Weijer, Univ. Dundee).

Dictyostelium shares many properties with animal cells but also has several features of plants. For instance, cellulose microfibrils reinforce the extracellular matrix after cells have aggregated, and form the stalk of the fruiting body⁵. The gene encoding the catalytic subunit of cellulose synthase has been difficult to pin down in plants, but has been discovered in a screen for *Dictyostelium* mutants that fail to make cellulose (L. Blanton, Texas Tech.). Mutant fruiting bodies are unable to support their own weight, and collapse soon after they form. Further studies have shown that there is a single gene for

cellulose synthase, making *Dictyostelium* an especially attractive test system for expression of putative plant cellulose synthase genes as well as for uncovering accessory proteins.

Medical interest in *Dictyostelium* will also be stimulated by the discovery that it can be infected by the intracellular bacterial pathogen *Legionella pneumophila* (J. Solomon, Tufts Univ.). In human lung macrophages and *Dictyostelium* amoebae, the bacteria escape from the phagocytic pathway, grow in vesicles inside the host cells, and eventually burst them. Genetic screens for resistant strains of *Dictyostelium* can now be used to uncover host functions manipulated by this stealth bacterium.

Although a characteristic of *Dictyostelium* research is its breadth, it must be seen in the round (J. Bonner, Princeton Univ.): "One must never lose what Barbara McClintock called, 'the feeling for the organism'. It makes no difference at what level one works, whether it be molecules or ecology or any level in between: all is lost if one forgets that *Dictyostelium* is an organism. In this way one can keep one's work in tune with nature; in this way one can see the all-important connections between all the levels of inquiry." ■

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*International Dictyostelium Conference, Bar Harbor, Maine, 14–19 August 1999. <http://www.sp.uconn.edu/~knecht/>

Daedalus

An actively cold wind

Wind-power, says Daedalus, is a mixed ecological blessing. Even a very windy site crowded with noisy turbines delivers only a modest amount of electrical power, rather unreliably. Much power these days goes to cool and air-condition buildings. Daedalus reckons the wind could do the job directly.

He points out that a guided air-stream can develop a temperature gradient, a fact exploited in the vortex-tube refrigerator. Air blown helically into a cunningly shaped tube divides into a hot stream and a cold one. The power for this thermodynamic feat comes from the motion of the air itself. So, says Daedalus, it should be possible to give a building a subtle aerofoil shape, such that the wind blowing past it cools it by the vortex effect, while discharging the waste heat into a separate stream downwind. Buildings with strange shapes are highly fashionable these days, and not only in Sydney and Bilbao. But could the design be practical? Commercial vortex-tube refrigerators use air at several atmospheres. Even a strong gale efficiently harnessed would produce less than a degree of cooling.

Daedalus is undaunted. He will give his building regenerative cooling. Banks of internal heat-pipes, with their almost infinite effective conductivity, will return the initial cooling to the incoming wind. The next cycle of cooling will be that much greater. This cooling too will be returned to the incoming wind, and so on. When the required degree of cooling has been reached, the heat pipes will be throttled back.

The shape to do all this will be subtle indeed. Daedalus's starting design echoes those chimneys whose helical vanes direct the wind upwards no matter from which direction it blows. The rising vortex will have its greatest cooling effect on the roof, from which heat-pipes will recycle the cold to incoming ground-level air again. By reversing the vanes, the building can intercept the heated airstream instead, thus allowing the wind actively to warm the building. With its large cross-section, the structure will capture far more energy than any turbine; and as a distributed heat pump, it will be able to deliver many joules of heat or cold for every joule of intercepted energy. The instantaneous heating or cooling effect will vary wildly with the gusting of the wind, but the thermal mass of the structure will smooth it out well. Daedalus's wind-conditioned buildings will be a major contribution to ecological engineering.

David Jones

Obituary

Ernst Ludwig Wynder 1922–99

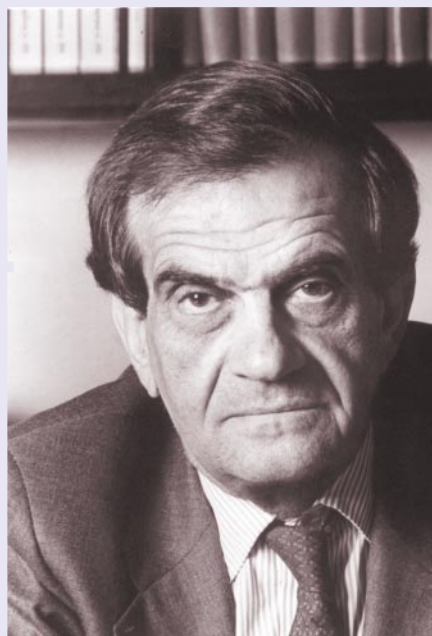
In the world of cancer researchers, a rather sober lot, Ernst Wynder stood out in brilliant colour. Wynder, who died earlier this year, was a pioneer who spent most of the past 50 years as a public-health crusader, preaching that cancer prevention would ultimately prove far more effective in reducing mortality than would new forms of treatment.

Wynder was born in Herford, Westphalia, Germany, but as the racial persecution of the late-1930s grew in intensity his family fled to the United States. After the war, he entered Washington University Medical School in St Louis, and, already very ambitious, laid out a plan for winning a coveted research prize for medical students. His idea came from an autopsy of a lung-cancer patient in which he happened to participate. He questioned the widow, discovered that the deceased had been a two-pack-a-day man for 30 years, and built on this clue by studying the histories of 20 other lung-cancer patients and 20 controls. By late 1948, he was able to assemble a preliminary report indicating that lung cancer was rife among smokers. And, of course, he won the research prize.

In fact, recognition of the connection between smoking and lung cancer was hardly new (as discussed, coincidentally, on page 425). Among its other admonitions, Nazi propaganda included a warning against the ill effects of smoking, and Hitler himself believed that tobacco was the root of almost all evil. A German study, published in 1939, indicated that non-smokers were more common in healthy populations than among lung-cancer patients.

But like much else that went on in Germany in the period 1933–45, this early epidemiological work was tainted by its Nazi associations and was ignored. For this reason, the connection between smoking and lung cancer remained a matter of intense debate long after the Second World War ended. Air pollution was at the top of the list of suspected causes.

Wynder's initial research was greeted by scepticism, even by the American Cancer Society. But by mid-1949, he and his mentor, Evarts Graham, had accumulated data on 648 cases of lung cancer and 600 normal controls. The study that they published in the *Journal of the American Medical Association* showed lung cancer to be as much as 40 times more common in heavy smokers than in non-smokers. Six months later, in Britain,



Epidemiologist and anti-smoking campaigner

Richard Doll published a more definitive prospective study on smoking and non-smoking groups of British physicians that reached the same conclusion.

By his own account, Wynder's work attracted little attention until he extended it through research at the Sloan-Kettering Institute in New York where he moved after his medical studies. In 1953, he showed that the condensate of cigarette smoke, when painted on the backs of mice, was a potent inducer of skin cancer. But not all were won over. In 1960, Wynder had a public debate with Clarence Cook Little, founder of the Jackson Laboratory in Bar Harbor, Maine, and a pioneer in the discovery of the mammary tumour virus of mice. Little, a pillar of the US cancer-research establishment, argued that viruses were the cause of human cancer, and that Wynder's epidemiology was at best correlative. The debate ended in a draw.

Wynder's time at Sloan-Kettering was not a tranquil one. The tobacco companies generously supported the institute's research work and wanted Frank Horsfall, its director, to muzzle Wynder. In the end, Wynder's freedom to continue proselytizing against smoking was ensured by Peyton Rous, virologist and soon-to-be Nobel laureate — Rous walked across the street from Sloan-Kettering's sister

institution, the Rockefeller Institute, and told Horsfall to back off.

There were also tensions between Wynder and his colleagues, who viewed his frequent mass-media diatribes against the evils of tobacco as self-promotion and questioned the rigour of his science. Wynder also had a side that seemed overly flamboyant to them: he was a *bon vivant*, a ladies' man. Long a bachelor, he was often seen about town with one or another young starlet on his arm.

Wynder had not endeared himself to his colleagues. With the winds at Sloan-Kettering blowing against him, he left in 1969 and founded the American Health Foundation (AHF), located today in Valhalla north of New York City. He built it from the ground up to a staff size of 200.

The agenda of the AHF was clear from the beginning: Wynder believed strongly that the most significant reductions in cancer mortality would come from changes in lifestyle. These views were already spelled out in 1952 in the mammoth 28-page study, published in the *New England Journal of Medicine*, entitled "Medical Progress: Some Practical Aspects of Cancer Prevention".

Research at the AHF centred on epidemiology and the use of animal models to follow up suspected connections between dietary intake and specific types of malignancies. In recent years, the foundation launched a programme in elementary schools that attempted to alter the life courses of young children by inculcating them with the virtues of healthy diet and smoke-free air. This was an exercise that Wynder felt would pay handsome dividends sometime in the mid-twenty-first century.

Wynder portrayed himself as being far removed from the mainstream of contemporary cancer research — the reductionist world of molecules and cells — and argued that it has far less to offer improvement of the human condition than epidemiology and public health measures. The world of science, he often said, had become too enraptured with the latest discovery in cancer genetics and had lost sight of its mandate to keep people healthy. He never retired, working unabated as head of the AHF until his death on 14 July this year, aged 77.

Ernst Wynder leaves behind an enormous, often unappreciated imprint on cancer research, and more than 750 papers on the epidemiology and aetiology of the disease. He is survived by his wife Sandra.

Robert Weinberg

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Evolution of an antifreeze glycoprotein

A blood protein that keeps Antarctic fish from freezing arose from a digestive enzyme.

The ice-binding antifreeze glycoprotein (AFGP) that circulates in the blood of Antarctic notothenioid fishes enables them to avoid freezing in their perpetually icy environment¹. This crucial survival protein probably arose from a functionally unrelated pancreatic trypsinogen-like protease². We have now discovered an important intermediate in this evolutionary process — transcriptionally active chimaeric genes that encode both an AFGP polypeptide and the protease, confirming the protease origin of AFGP and indicating how it was created.

AFGP binds to and arrests the growth of ice crystals that enter the fish, thereby preventing the fish from freezing. There are at least eight forms of the protein of different sizes (AFGP 1–8), all composed of repeats of a simple glycotripeptide monomer (Thr-Ala/Pro-Ala-) with a disaccharide attached to each threonine residue¹. These isoforms are encoded as distinct molecules linked in a series by conserved three-residue spacers (mostly Leu/Phe-Ile/Asn-Phe) within a large polypeptide; post-translational removal of the spacers produces many copies of mature AFGP per gene^{2,3}. A typical AFGP gene comprises two exons and a single intron, with the small exon (E1) encoding the signal peptide and the large exon (E2) encoding the AFGP polypeptide (Fig. 1a).

All notothenioid AFGP polypeptide genes characterized so far encode only the small isoforms (AFGPs 6, 7 and 8)^{2,4}. To determine whether the large isoforms (AFGPs 1–5) are also individually encoded, we screened a genomic library obtained from *Dissostichus mawsoni* (the giant Antarctic toothfish) and isolated a clone, *Dm7M*, that contains an unusual gene encoding not only the large AFGP isoforms but also a trypsinogen-like protease in tandem (Fig. 1c, middle).

The chimaeric gene in clone *Dm7M* spans about 9.6 kilobase pairs (kb), which we sequenced completely. It is a partial gene lacking its 5' end, which we subsequently obtained by amplification of genomic DNA using *Dm7M*-specific primers in the polymerase chain reaction (PCR) (Fig. 1c, top left). The complete reconstructed chimaeric gene (~11 kb) consists of six exons and five introns, and its arrangement is identical to that of independent trypsinogen-like protease genes² (Fig. 1b), with the notable exception of a hybrid exon 2 (Fig. 1c, middle). Hybrid E2 has a large 5' AFGP polypeptide-coding segment that encodes seven AFGP molecules (two large and five

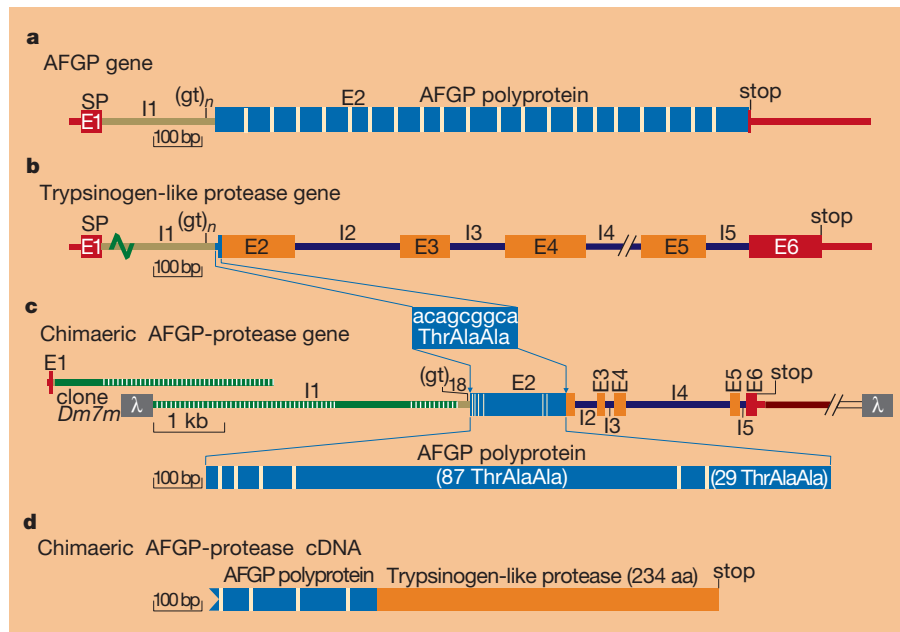


Figure 1 Key molecular components in the evolution of the AFGP gene from the protease gene in Antarctic notothenioids. **a**, An independent AFGP polypeptide gene; blue, AFGP coding sequences; pink, spacer sequences. SP, signal peptide. **b**, An independent trypsinogen-like protease gene. **c**, Chimaeric AFGP-protease gene. PCR amplification of genomic DNA produced the 5' sequence (top left) of the partial chimaeric gene in clone *Dm7m* (middle), which is expanded at the hybrid exon E2 to show the AFGP polypeptide (bottom). **d**, Chimaeric AFGP-protease cDNA from a chimaeric gene distinct from *Dm7M*. Homologous sequence segments have identical colours. The notothenioid AFGP gene arose from recruitment of the front (exon E1 and intron I1) and the tail (E6) of an ancestral protease gene, *de novo* creation of the AFGP coding region by repeated duplications of the Thr-Ala-Ala-coding element that straddles the I1–E2 junction of the protease gene (enlarged between **b** and **c**), and deletion of the bulk of the protease sequence (E2–I5). This transcriptionally active chimaeric AFGP-protease gene confirms the protease origin of the AFGP gene and indicates how it evolved.

small isoforms) (Fig. 1c, bottom), and a small 3' segment that is similar in sequence to the trypsinogen-like protease exon 2 reported previously².

The location of the AFGP-polypeptide-coding segment in the chimaeric gene corresponds to that of the single Thr-Ala-Ala-coding element in the protease gene (this element straddles the junction of E2 with the first intron (I1))² (Fig. 1b), which strongly indicates that the repetitive AFGP-polypeptide-coding sequence arose from expansion of the element (together with a spacer whose origin is unknown) through iterative duplications. The chimaeric-gene intron-1 sequence, apart from two insertions, is very similar to that of the distinct AFGP genes, including the (gt)_n-minisatellite DNA-bearing I1 sequence that was apparently inherited from the ancestral trypsinogen-like protease (this (gt)_n sequence persists in the present-day trypsinogen-like gene; Fig. 1b,c). This (gt)_n sequence presumably facilitated the first duplication of the ancestral Thr-Ala-Ala-coding element through an accidental replication slippage^{5,6}. Two of the tripeptide repeats may have partial ice-binding activity (the smallest functional isoform, AFGP8,

has only four repeats) and as the Antarctic water chilled towards freezing, they perhaps became selected upon for *de novo* expansion through further replication slippage^{5,6} or unequal crossing over⁷.

The *Dm7M* chimaeric AFGP-protease gene is probably not a pseudogene, as it has all the expected elements of a functional gene, and similar chimaeric genes in the toothfish are found to be transcribed. We obtained partial AFGP-protease chimaeric complementary DNAs by using reverse transcription with PCR of toothfish pancreatic messenger RNA (Fig. 1d). The encoded partial AFGP polypeptide contains four AFGP molecules, followed without interruption by the entire trypsinogen-like protease. This AFGP polypeptide differs from that encoded in clone *Dm7M* (Fig. 1a), indicating that there is more than one chimaeric AFGP-protease gene in the toothfish genome. We found by Southern-blot analysis of genomic DNA that chimaeric AFGP-protease genes also exist in other notothenioid species (results not shown).

The simultaneous presence of a present-day protease gene carrying the incipient Thr-Ala-Ala-coding element, a transcriptionally

active chimaeric AFGP–protease gene, and independent AFGP genes in the toothfish genome confirms that the AFGP sequence started out as a small integral part of the ancestral protease gene, underwent expansion, and acquired independence by shedding the bulk of the protease sequence (E2 to I5)². By capturing the chimaeric evolutionary intermediates, we have a rare view of the genesis of a new protein that ultimately enabled the Notothenioid suborder to rise to dominance in the freezing Southern Ocean^{8,9}.

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Animal behaviour

Aquatic sex pheromone from a male tree frog

Many creatures use chemical signals (pheromones) as sources of information about the world around them^{1–3}. For example, a sex pheromone produced by one sex (usually the female) of a particular species induces an immediate behavioural response in the opposite sex of the same species^{2,3}. However, very little is known about amphibian pheromones⁴. We have now discovered and characterized an aquatic, female-attracting pheromone from the parotoid and rostral glands of a male frog, *Litoria splendida*. To our knowledge, this pheromone, which we have named splendipherin, is the first

pheromone from an anuran (frog or toad) to be identified.

The skin glands of anurans secrete defence compounds, including many types of biologically active peptides^{5,6}. For example, *L. splendida* (Fig. 1) exudes several biologically active peptides from the parotoid and rostral glands (situated at the rear and front of the head, respectively), including the broad-spectrum antimicrobial caerin-1 peptides (Fig. 2)^{7,8}. However, no anuran pheromones have been reported, apart from a possible alarm pheromone in tadpoles of the toad *Bufo bufo*⁹.

Because anurans often breed in aquatic conditions and deposit their eggs in water, it is possible that they produce peptides as water-soluble aquatic pheromones. This proposal is supported by the identification of the sex pheromone sodefrin (a decapep-



Figure 1 The magnificent tree frog, *Litoria splendida*.

tide, SIPSKDALLK-OH) from the cloacal gland of the male newt *Cynops pyrrhogaster*¹⁰.

We monitored the peptide content of secretions from the parotoid and rostral glands of male and female *L. splendida* every month for three years by using high-performance liquid chromatography and electrospray mass spectrometry. No animals were killed during this procedure¹¹. A minor component that is found only in male secretions can be seen in the partial HPLC profile shown in Fig. 2. This peptide, which we call splendipherin, comprises 25 amino-acid residues with the sequence GLVSSIGKALGGLADVVKSGQPA-OH.

The concentration of splendipherin peaks during the breeding season (January to March) to about 1% of the total peptide content of the glandular secretion, and decreases tenfold during June to November. Unlike the other peptides in Fig. 2, splendipherin has no antimicrobial activity. The natural peptide contains L-amino acids, as confirmed by the preparation of synthetic L-splendipherin, which has the same pheromone activity as the natural material.

The pheromone behavioural tests were carried out in a glass tank (0.65 × 2.0 × 0.75 metres) containing water 2 cm deep. Female *L. splendida* frogs were placed in the centre of the tank, where they remained for up to ten minutes without moving. Adding 40 nanograms of splendipherin in water (a final concentration of 10^{–13} M) to a gauze pad 1 m from the frog elicited a distinct change in posture and increased alertness in the frog within 20 seconds. The female then walked slowly towards the pad, sat on it, and remained in a sitting position until she was removed. This experiment has been repeated several times and in different directions, using eight different females, with a 100% success rate.

The average time between introducing the pheromone and the female adopting a sitting position on the gauze pad is 7 minutes. The speed of the initial recognition indicates that splendipherin may be acting as a surfactant, but this has yet to be confirmed experimentally. When more than 4,000 nanograms of pheromone is added to the pad, the female becomes confused and

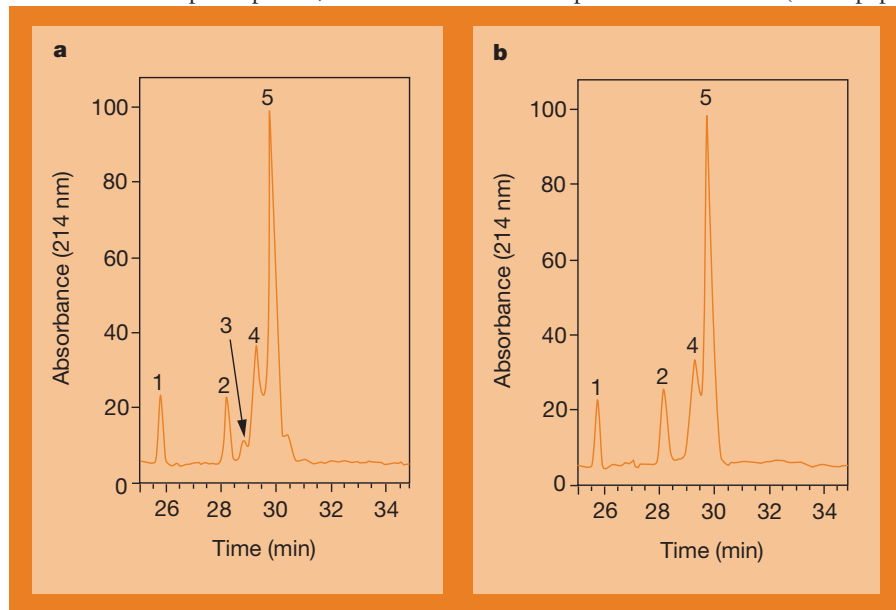


Figure 2 Partial high-performance liquid chromatography (HPLC) traces of the glandular secretions of *Litoria splendida*. a, Male; b, female. For experimental conditions, see refs 8,12. The components are as follows: 1, GLWQIKDKASELVSGIVEGVK-NH₂ (caerin 3.1; antibiotic)¹²; 2, GLVSSIGKALGGLADVVKSGQPA-OH (caerin 2.1; weak antibiotic)¹²; 3, GLVSSIGKALGGLADVVKSGQPA-OH (splendipherin); 4, GLFSLGAVAKHVLPHWVPIAEKL-NH₂ (caerin 1.6; antibiotic)⁸; 5, GLVSSIGKALGGLADVVKSGQPA-OH (caerin 1.1; antibiotic)^{7,8}. For the full HPLC trace, see ref. 12. Caerin 2.1 differs from splendipherin in that an arginine residue replaces a lysine residue at position 8. Caerin 2.1 has no pheromone activity towards either male or female *L. splendida*.

is unable to find the source, whereas just 4 nanograms elicits no response. In similar pheromone tests, splendipherin does not elicit a response from male *L. splendida* or either males or females of the closely related tree frog *L. caerulea*. We conclude that the aquatic, male sex pheromone splendipherin is therefore species specific.

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Protein structure

An enzymatic globin from a marine worm

Some marine worms, such as *Thelepus crispus* and *Notomastus lobatus*, secrete brominated aromatic molecules and other halogenated metabolites as repellants¹. Other species, such as *Amphitrite ornata*, do not produce repellants but are adapted to the chemical warfare of *N. lobatus* and cohabit with them in estuarine mudflats. The halocompounds are tolerated by *A. ornata* as they are degraded by dehaloperoxidase (DHP)². We have determined the amino-acid sequence and crystal structure of DHP and find that its fold is typical of the globin family, indicating that the enzyme evolved from an oxygen carrier protein. Residues at the dimer interface do not correspond to those in tetrameric and dimeric haemoglobins and the spatial arrangement of the dimers is different. The complete amino-acid sequence is most similar to that of myoglobin from the sea hare, with 20.6% identity among 126 overlapping amino acids.

The halophenols bind in the distal pocket of DHP, indicating that DHP removes the halogen by directly attacking an activated

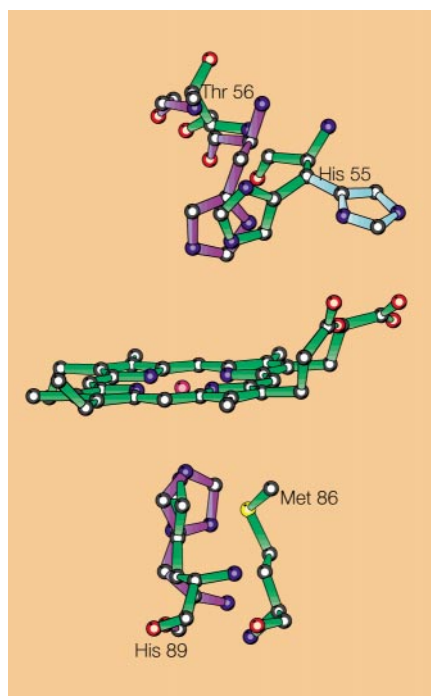


Figure 1 The least-squares superposition of the haem groups of dehaloperoxidase (green) and myoglobin (Protein Data Bank entry code, 1mbo), showing the most important differences in their environment. The position of the distal histidine of dehaloperoxidase out of the binding pocket is shown in cyan, and myoglobin residues are purple.

oxygen atom. If this is the case, then DHP represents a new class of enzyme: a dehaloperoxygenase. Its mechanism of action is probably similar to that of other peroxidases, given the important role of the high-valency iron–oxo intermediate that is formed when hydrogen peroxide is added to the enzyme and the subsequent heterolytic cleavage of the O–O bond³. The ability of peroxidases to form such an intermediate, which globins cannot, is consistent with the idea⁴ that the proximal histidine and polar residues in the distal pocket are crucial.

Peroxidases form a strong hydrogen bond between the N₈₁ nitrogen atom of the proximal histidine and a nearby glutamate, so the proximal histidine takes on a partial histidinate character. In contrast, the proximal pocket of globins lacks a glutamate, so they form only a weak hydrogen bond⁴. In DHP, a hydrogen bond forms that is stronger than in globins but, rather than bonding to a carboxylate, it is generated by a roughly 60° rotation of the imidazole ring. Its hydrogen points directly into the oxygen atom of the peptide carbonyl group of the leucine at residue 83. The hydrogen bond is shorter than that of myoglobin, in which it is bifurcated (Fig. 1). The reorientation of the axial ligand is facilitated by a shift of the proximal histidine in the sequence by two residues.

In peroxidases, the distal histidine functions as an acid or base in the transfer of

protons to the leaving water molecule, and the developing negative charge is stabilized by an arginine side chain⁴. This arrangement is possible because peroxidases do not use the distal cavity as an organic-substrate-binding pocket, as the reaction takes place at the haem edge⁵.

We propose that DHP binds peroxide and uses the distal histidine to cleave the O–O bond. When the high-valency iron–oxo intermediate is ready, the distal histidine swings out of the cavity, enabling the substrate to enter the distal pocket and undergo oxidation. This hypothesis is supported by the observation that the distal histidine in the native DHP is disordered between two positions, with one outside the distal pocket and the other inside (Fig. 1), whereas it is outside the pocket in the complex with 4-iodophenol.

The distance between the N₆₂ atom of the distal histidine and the haem iron in globins is 4.1 to 4.6 Å, whereas in peroxidases it is 5.5 to 6.0 Å (ref. 6). In DHP, this distance is 5.4 Å for the position inside the distal cavity, which is related to a 2.4 Å shift of its α -carbon compared with myoglobin. The shift appears to be generated by the replacement of the glycine that follows the distal histidine in myoglobin by threonine in DHP.

DHP serves primarily to dehalogenate biologically generated bromophenols, but it can also dehalogenate trichlorophenols and other anthropogenic pollutants. The efficiency of the enzyme is not significantly affected by the position and number of halogen atoms at the phenol ring². The enzyme simply binds its organic substrate close to a highly reactive oxygen atom and so it does not need a system involving hydrogen bonds and other interactions that are features of enzymes with more sophisticated mechanisms. This mechanism means that its specificity can be altered by modifying the residues lining the distal cavity, making it potentially useful as a tool for bioremediation projects.

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Lake ecosystems

Rapid evolution revealed by dormant eggs

Natural selection can lead to rapid changes in organisms, which can in turn influence ecosystem processes¹. A key factor in the functioning of lake ecosystems is the rate at which primary producers are eaten, and major consumers, such as the zooplankton *Daphnia*², can be subject to strong selection pressures when phytoplankton assemblages change. Lake Constance in central Europe experienced a period of eutrophication (the biological effects of an input of plant nutrients) during the 1960s–70s³, which caused an increase⁴ in the abundance of nutritionally poor or even toxic⁵ cyanobacteria. By hatching long-dormant eggs⁶ of *Daphnia galeata* found in lake sediments, we show that the mean resistance of *Daphnia* genotypes to dietary cyanobacteria increased significantly during this eutrophication. This rapid evolution of resistance has implications for the ways that ecosystems respond to nutrient enrichment through the impact of grazers on primary production.

Genetically distinct³ *Daphnia galeata* clones were collected as diapausing eggs from sediments of known age³ obtained from a core taken from Lake Constance in 1997. Parthenogenetic lines were maintained for each clone for at least 40 generations to minimize maternal effects. We tested 32 clones from three sediment ages for their resistance to dietary cyanobacteria: 12 clones from 1962–64 and 1969–71 (before and just after the appearance of cyanobacteria; Fig. 1), 10 clones from 1978–80 (peak eutrophication), and 10 clones from 1992–94 and 1995–97 (when the period of eutrophication had passed).

Resistance to cyanobacteria was measured as the effect of diet on the somatic juvenile growth rate (g) of *Daphnia*: $g = [\ln(W_m) - \ln(W_n)]/t$, where W_m and W_n are the masses of mature and neonate *Daphnia*, respectively, and t is the development period. For each clone, g was measured for animals fed two different diets (supplied at 1 mg carbon per litre): one diet (g_{poor}) contained a mixture of a toxic cyanobacterium (*Microcystis aeruginosa*, 20% by carbon content) and a high-quality algal resource (*Scenedesmus obliquus*, 80% by carbon content); the other diet (g_{good}) contained only *Scenedesmus*. *Microcystis* was originally isolated from Lake Constance in 1972, is toxic to *Daphnia pulex*⁸ and contains high concentrations of microcystin-LR hepatotoxin, whereas *Scenedesmus* promotes growth and reproduction in *Daphnia*⁶. *Microcystis* and *Scenedesmus* were fed to *Daphnia* as single cells of similar size (4.2 μm).

For each clone, g_{good} and g_{poor} were measured separately by using three replicate

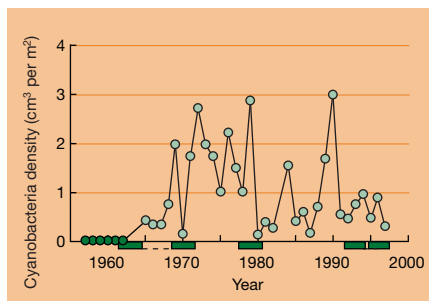


Figure 1 The summer density of planktonic cyanobacteria (total cell volume) in Lake Constance⁴. Filled circles, years when cyanobacteria were recorded as extremely rare; thick bars, sediment ages from which dormant *Daphnia* eggs were studied; the dashed line connects time periods analysed together.

flow-through vessels (5–17 individuals each) with food levels in excess of growth-limiting concentrations⁹. Because there was variation among clones in g_{good} (likelihood ratio test, $\chi^2 = 120.1$, d.f. = 1, $P < 0.0001$), resistance to dietary cyanobacteria was standardized as the growth-rate reduction, R , which is the fractional reduction in g on poor food relative to that on good food: $R = (g_{\text{good}} - g_{\text{poor}})/g_{\text{good}}$.

The *Daphnia* population evolved an increased ability to cope with a diet containing cyanobacteria (Fig. 2). Genotypes from both 1978–80 and the 1990s exhibit lower growth-rate reduction than those from 1962–64 and 1969–71 (ANOVA planned contrasts, $F = 10.3$, d.f. = 1, 29, $P = 0.003$, and $F = 11.9$, d.f. = 1, 29, $P = 0.002$, respectively). The rapid response observed during 1969–80 is attributable entirely to a reduction in genetic variance (likelihood ratio test, $\chi^2 = 8.24$, d.f. = 1, $P = 0.002$). There was a broad range of *Daphnia* genotypes of different growth-rate reductions in the lake during 1962–64 and 1969–71, but the genotypes that were most heavily affected by dietary cyanobacteria were virtually eliminated within ten years of continued summertime exposure to cyanobacteria.

The mean resistance of *Daphnia* to cyanobacteria was unchanged between 1978–80 and the 1990s (ANOVA planned contrast, $F = 0.06$, d.f. = 1, 29, $P = 0.82$). However, the variance of growth-rate reductions was greater in the 1990s than in 1978–80 (likelihood ratio test, $\chi^2 = 4.72$, d.f. = 1, $P = 0.015$), perhaps as a result of the water column being reinvaded by animals hatching from the dormant egg bank⁶.

These short-term evolutionary changes may significantly affect the course of ecosystem change. Greater abundance of cyanobacteria during eutrophication is typically considered to be a response to increased nutrient inputs¹⁰. However, rapid adaptive evolution in grazing zooplankton populations may be an important feedback mechanism that is critical to understanding

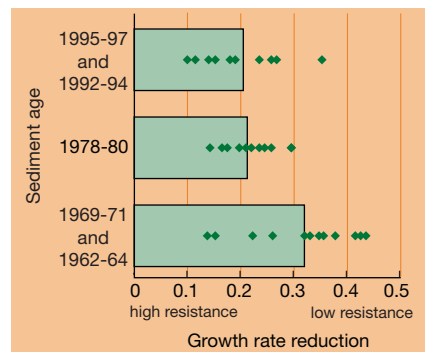


Figure 2 Resistance of *Daphnia* genotypes to dietary cyanobacteria. Data points represent estimates of growth-rate reduction for individual clones.

the net effect of eutrophication on primary producers in lakes.

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Correction

Food contamination by PCBs and dioxins

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Nature **401**, 231–232 (1999)

The average ratio of polychlorinated biphenyls (PCBs) to polychlorodibenzodioxins (PCDDs) or polychlorodibenzofurans (PCDFs) in samples of contaminated feedstuff, chicken meat and eggs (Fig. 1b) was incorrect as published (see fourth paragraph): the average ratio of PCB:PCDD/F was about 50,000:1 (not 22,000:1). The conclusions of our report are unchanged.

The origins of insect metamorphosis

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Insect metamorphosis is a fascinating and highly successful biological adaptation, but there is much uncertainty as to how it evolved. Ancestral insect species did not undergo metamorphosis and there are still some existing species that lack metamorphosis or undergo only partial metamorphosis. Based on endocrine studies and morphological comparisons of the development of insect species with and without metamorphosis, a novel hypothesis for the evolution of metamorphosis is proposed. Changes in the endocrinology of development are central to this hypothesis. The three stages of the ancestral insect species—pronymph, nymph and adult—are proposed to be equivalent to the larva, pupa and adult stages of insects with complete metamorphosis. This proposal has general implications for insect developmental biology.

Metamorphosis is one of the most widely used life-history strategies of animals. The dramatic differences between larval and adult forms allow the stages to exploit different habitats and food sources, and also allow the extreme adaptation of one stage for a particular role, such as dispersal. In amphibians and many marine invertebrates, metamorphosis is an ancestral condition and its origins are buried deep in the evolution of these groups. In insects, however, the earliest forms showed direct development (were ametabolous) and the evolution of metamorphosis then fuelled their dramatic radiation^{1,2}. The earliest true insects included some of the ametabolous orders that are still present today, the bristletails and the silverfish. Their juvenile stages look very much like the adult, except that they lack functional genitalia. Insects with 'incomplete' metamorphosis (hemimetabolous insects) are a polyphyletic assemblage which includes cockroaches, grasshoppers, dragonflies and true bugs. Their immature stages were originally called nymphs, and we will use that terminology here. Besides lacking genitalia, the nymphs of winged species bear wing buds folded over their backs, which are finally transformed into articulated, functional wings during the moult to the adult stage. Insects with 'complete metamorphosis' were first seen in the Permian and constitute a monophyletic group³, the Holometabola (including beetles, flies, moths and bees). They have very different larval, pupal and adult stages, which allows them to separate the resources needed for growth from those needed for reproduction. They have also achieved extremely rapid life cycles.

How did the three-part life cycle that characterizes the Holometabola evolve from the nymph and adult stages of more basal insects? Two opposing hypotheses have been advanced. The first was formulated by Berlese⁴, who noted a similarity between different larval body forms and the morphological transitions seen during embryogenesis of hemimetabolous insects. He proposed that the holometabolous larva arose by a process of 'de-embryonization' so that the larva was essentially a free-living, feeding embryo. The premature hatching was thought to be caused by a reduction in the amount of yolk stored in the egg, and hatching at different times generated a diversity of larval forms. As the larva took over the feeding responsibilities, the nymph was reduced to a single instar that became the pupa.

The alternative hypothesis for metamorphosis held that larvae and nymphs were equivalent, and that the pupal stage arose *de novo*, as the disparity between larva and adult widened^{5,6}. Proponents of the latter hypothesis claimed that there was no difference in the amount of yolk in the eggs of holometabolous and hemimetabolous insects⁷, and that some larval specializations, such as the abdominal prolegs of certain scorpionfly larvae, were derived structures that did not arise from the embryonic appendages⁸. The latter hypothesis, considering larvae and nymphs as equivalent stages, has been more widely followed^{9,10}. Our examination of the endocrine control

of embryonic and postembryonic development, though, suggests that the roots of metamorphosis are to be found in embryonic stages, more in line with the views of Berlese, and as recognized by the Czechoslovakian insect physiologist, V. Novak¹¹.

Form and function of the pronymph stage

In many arthropods, the stage that hatches from the egg has unique features that distinguish it from subsequent life stages. This is quite obvious in aquatic and marine crustaceans that have a specialized nauplius stage which serves as a primary dispersal stage. A unique hatchling stage is also seen in terrestrial arthropods, such as isopods

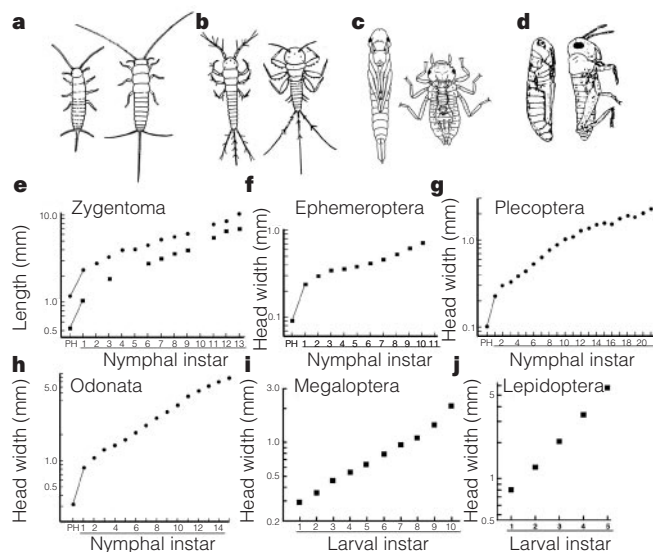


Figure 1 Comparison of the early immature stages of ametabolous, hemimetabolous and holometabolous insect species. **a–d**, Drawings of the pronymph (left) and first-instar nymph (right) of ametabolous (**a**) and hemimetabolous (**b–d**) insects: **a**, the silverfish *Ctenolepisma longicaudata*; **b**, the mayfly *Stenonema interpunctatum*⁵⁵; **c**, the dragonfly *Epiophlebia superstes*⁵⁶; and **d**, *L. migratoria*²⁰. **e–j**, Comparison of the dimensions of the head or body parts of the immature instars of various insects during postembryonic life. In the ametabolous (**e**) and hemimetabolous (**f–h**) species, there is a marked shift in body proportions between the pronymph (PN) and the first nymphal instar. Typically, no such shift is seen in the immature series of holometabolous species (**i, j**). **e**, Lengths of the antenna (circles) and the cercal filaments at the end of the abdomen for the *C. longicaudata* (Zygentoma)¹⁴. **f**, Head width of *S. interpunctatum* (Ephemeroptera)⁵⁵. **g**, Head width of a stonefly, *Neoperla clymene* (Plecoptera)⁵⁷. **h**, Head width of the dragonfly *Anax imperator* (Odonata)⁵⁸. **i**, Head width of the alderfly *Sialis rotunda* (Megaloptera)⁵⁹. **j**, Head width of the tobacco hornworm *M. sexta* (Lepidoptera). The *Manduca* data are from measurements of 10 individuals; s.e.m.s were smaller than the data points.

(Crustacea), arachnids and myriapods, although it is not adapted for dispersal. It typically does not feed, and is often helpless and the object of maternal care (for example, in scorpions and woodlice^{12,13}). It subsists on its yolk supply and represents a continuation of embryonic development, but outside the confines of the egg shell. The first postembryonic moult finally transforms it into an active, feeding juvenile.

Our hypothesis for the evolution of metamorphosis focuses on a corresponding stage in the ametabolous and hemimetabolous insects. This is a largely ignored stage that we will call the pronymph. In the ametabolous insects, such as silverfish, it lasts for 3 to 4 days after hatching¹⁴, but in the hemimetabolous groups the pronymph becomes less obvious. In basal hemimetabolous orders (mayflies, dragonflies, stoneflies and grasshoppers), the pronymph stage is passed primarily in the egg and lasts for only minutes to a few hours after hatching¹⁵. In more derived hemimetabolous orders (such as true bugs and lice), the shedding of the pronymphal cuticle occurs during hatching and it is the first-stage nymph that actually emerges from the shell.

The pronymph has a number of characteristics that make it unique¹⁵. Its bodily proportions differ from those of the nymph (Fig. 1a–h) and probably reflect a developmental compromise for growing long appendages within the confined space of the egg shell. Its cuticle has a distinctive ultrastructure¹⁶ and lacks the rigid, tanned plates (sclerites) that characterize nymphal and adult cuticles. Its mandibles are typically unsclerotized, and it may possess specialized hatching devices such as 'egg bursters'. It lacks the wing buds that feature prominently in the growing nymph and, as detailed below, its endocrinology is distinct from that of the nymph. The embryo begins to secrete its pronymphal cuticle around the time of dorsal closure^{17–19} when it has achieved its full size (at about 48–60% of embryogenesis; %E). Although the embryo may have secreted cuticle before this time, the pronymphal cuticle is the first one that is sufficiently tough to require coordinated muscular activity for its shedding. The first nymphal cuticle is finally deposited at 75–85%E.

We propose that the hemimetabolous pronymph has become the holometabolous larva. This proposed relationship is reflected in a number of similarities between the two stages. (1) In holometabolous embryos, the secretion of the first-instar larval cuticle^{20–22} begins around the time of dorsal closure (45–55%E), which would correspond to the time of pronymphal cuticle secretion in hemimetabolous forms. A late bout of cuticle production, which would correspond to the nymphal moult^{17,19}, is absent in the Holometabola. (2) The larval body cuticle is typically soft and lacks sclerites, similar to that of the pronymph. (3) The larval sensory nervous system also shows similarities to that of the pronymph. At the beginning of its pronymphal stage, a grasshopper embryo has a stereotyped set of early-born neurons in each body segment²³ and appendage²⁴. Some of these neurons are 'pioneer' neurons that provide the pathways that are later used for the growth of nymphal sensory axons into the central nervous system (CNS). Importantly, the sensory neurons in the body wall of newly hatched larvae of both *Drosophila* and the moth *Manduca sexta* correspond to this early set of cells^{23,25}. Similarly, we think that the small number of sensory neurons that are found in the appendages of larvae are homologous to the set of pioneer neurons present at the start of the pronymphal stage in hemimetabolous species. In the pronymph, some of these neurons apparently die after completing their pioneering function²⁶. In the larva, in contrast, we expect that these cells survive as functional, larval sensory neurons. They may, however, eventually die at metamorphosis after they help to guide the adult sensory axons into the CNS. Therefore, rather than being a derived condition²⁷, the reduced sensory systems of holometabolous larvae seem to be a primitive condition that they share with the pronymph.

A major difference between a pronymph and a larva is that the former lasts for a single instar before transforming into the nymph (Fig. 1e–h), whereas, with the exception of some derived parasitic species, the latter maintains its form through successive instars until finally reaching the pupal stage (Fig. 1i, j). A shift in hormone secretion during embryogenesis may have been instrumental in

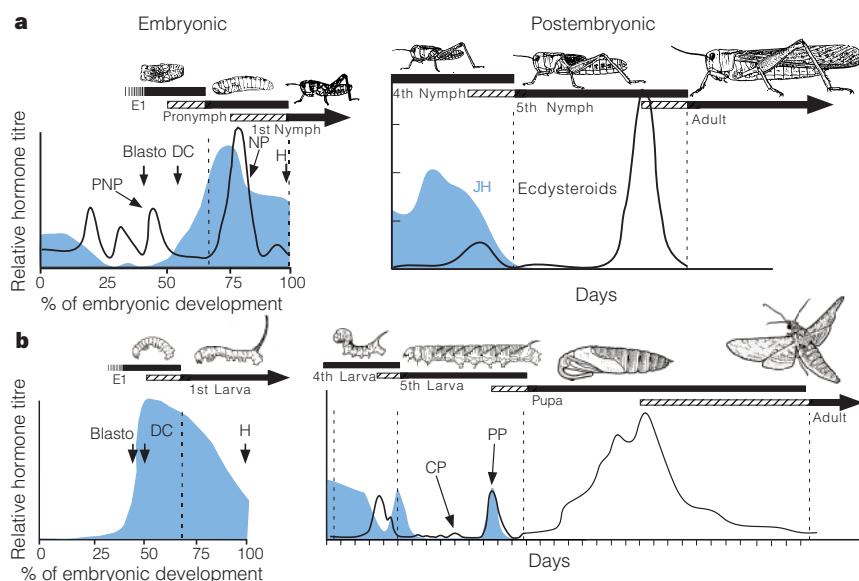


Figure 2 Endocrinology of embryonic and post-embryonic insect development. Comparison of the embryonic and postembryonic titres of ecdysteroids (black) and juvenile hormone (blue) for **a**, hemimetabolous insects, the grasshoppers *L. migratoria* (embryonic) and *Schistocerca gregaria* (postembryonic) and for **b**, a holometabolous insect, the sphinx moth *M. sexta*. The bars over the hormone titres show the times when the cuticles of the particular instars are present; cross-hatching represents the pharate stage when the insect is still covered by the old cuticle of the preceding stage. Detailed

ecdysteroid titres are not available for embryos of *Manduca*. Vertical dashed lines show the times of ecdysis (shedding of the old cuticle); Blasto, blastokinesis; DC, dorsal closure; E1, first embryonic instar; H, hatch. Ecdysteroid peaks: CP, commitment peak; PP, prepupal peak; PNP, pronymphal peak; NP, nymphal peak. The *Schistocerca* titre references are cited in ref. 60; more recent citations are supplied for *Locusta* showing embryonic ecdysteroid³⁰ and JH³¹ titres, and *Manduca* showing embryonic JH titres³⁷ and postembryonic titres for ecdysteroids⁶¹ and JH^{62,63}.

changing the transitional pronymphal stage into a stable larval stage.

Endocrinology of pronymph formation

Two families of hormones, the ecdysteroids and the juvenile hormones (JH), control moulting and metamorphosis during postembryonic life²⁸. The former induce the production of a new cuticle during a moult, whereas the latter regulate the character of that moult. For both larvae and nymphs, moults that occur in the presence of JH are 'status quo' moults that result in a new instar which retains its former morphology²⁹. The withdrawal of JH then allows metamorphosis to ensue. In hemimetabolous insects, the first moult after the decline of JH is to the adult (Fig. 2a). In holometabolous insects, by contrast, the endocrine interactions are more complex (Fig. 2b). After the initial disappearance of JH, a small peak of ecdysteroids 'commits' certain tissues to pupal differentiation²⁹. The pupal moult, though, is induced only later in response to a large surge of ecdysteroids. JH reappears during this ecdysteroid surge but then disappears before the large ecdysteroid peak that drives adult differentiation. The origin of this pattern of JH secretion has been unclear.

These hormones are also involved in the embryonic development of insects. In accord with its postembryonic function, peaks of ecdysteroids are also seen during the embryonic moults³⁰ (Fig. 2a). The JH titre, though, is unusual. For example, in the grasshopper *Locusta migratoria*, JH is found in newly laid eggs but disappears before the first embryonic moult and the initial phases of the pronymphal moult³¹. It then rises to its highest levels during nymph formation. Data from the embryos of other hemimetabolous insects, such as cockroaches³² and true bugs³³, also show low or no JH at the start of the pronymphal stage followed by high JH levels during nymphal differentiation.

That a JH-free period is essential for normal embryogenesis was shown by applying JH or JH mimics to embryos of hemimetabolous insects³⁴. In contrast to its 'status quo' action during postembryonic life, JH acts embryonically to promote precocious maturation. For

example, our treatment of embryos of *L. migratoria* with the JH mimic pyriproxyfen just before the pronymphal moult redirected development to produce precocious nymphs that had sclerotized mandibles, cuticular bristles and wing buds (Fig. 3a, b). Their body proportions fit these extrapolated for a '0'-instar nymph (Fig. 3c–f). Similarly treated embryos produced cuticle with a nymphal ultrastructure³⁵. The developmental window during which treatment with JH can induce a precocious nymph closes just before dorsal closure, after the pronymphal moult has commenced. Treatment with JH mimics very early in embryogenesis (at 15–20%E) resulted in tiny nymphs (Fig. 3b, right) bearing wing buds and having sclerotized mandibles appropriate for a '–1'-instar nymph. Importantly, both the '0'- and '–1'-instar nymphs were terminal embryonic stages that showed no further moulting.

An alternative approach is to deny developing embryos their normal JH by treating them with precocenes to destroy the embryonic corpora allata, the source of JH. In both cockroaches³⁶ and crickets (*D. Erezylmaz, L.M.R. & J.W.T.*, unpublished results), such treated embryos eventually became nymphs but the maturation of their internal tissues was severely suppressed. Thus, in these hemimetabolous embryos, JH seems to be involved in regulating the shift between growth and tissue maturation.

In the Holometabola, detailed embryonic JH titres are available for only a few species of moths³⁷. Interestingly, these embryos already show a high JH titre when they initiate their moult at dorsal closure. This precocious appearance of JH is probably associated with this moult's now leading to a fully differentiated form—the larva—rather than to the transitional pronymph stage of hemimetabolous insects.

Application of exogenous JH or JH mimics to embryos of flies and moths has only minor effects on embryonic growth and morphogenesis, although it does interfere with some morphogenetic movements such as blastokinesis^{38–40}. We think that these holometabolous embryos are relatively insensitive to exogenous JH because much of the embryonic growth that was ancestrally sensitive to JH has been shifted into postembryonic life during the evolution of the larval form.

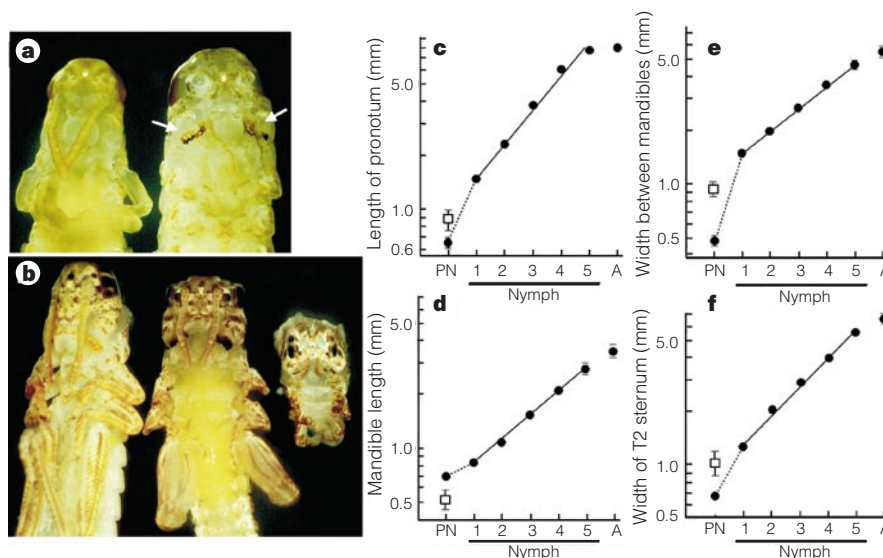


Figure 3 The effects of the JH mimic pyriproxyfen on *Locusta* embryos. **a**, A ventral view of the anterior end of embryos three days after treatment with acetone (left) or pyriproxyfen (right) at about 47%E. The acetone-treated embryo is a newly ecdysed pronymph (~68%E). Its clutchmate, treated with the JH mimic, shows sclerotized mandibles (arrows) and the dimensions of a head and thorax predicted for a '0'-instar nymph. **b**, Ventral views of (left) control embryo about 1 day before hatching, (middle) a '0'-instar nymph of comparable age produced by JH mimic treatment before dorsal closure, and (right) a '–1'-instar nymph formed after JH mimic treatment before blastokinesis.

c–f, Quantification of the result of treating embryos with the JH mimic at 45%E. The normal progression of the size of various structures between the pronymph (PN) and nymphal instars is represented as in Fig. 1. Points are the means ± s.e.m. for 10 individuals; s.e.m.s smaller than the data points are not shown. The treatment with the JH mimic (squares, ± s.e.m. for $n = 14$) redirected the pronymphal moult to produce animals with proportions that were shifted towards those predicted for a hypothetical '0'-instar nymph. Similar results are seen after treatment with physiological dosages of JHIII.

JH and postembryonic development

At first glance, the effects of JH in promoting tissue maturation in hemimetabolous insect embryos seem contrary to its 'status quo' action during postembryonic life. The action of JH on embryos, though, might be better contrasted with its action on imaginal discs—tissues that are in an 'embryonic' condition in the larva and will give rise to the structures of the adult. Over the years most of the studies of imaginal discs have focused on Lepidoptera and higher flies. In these insects the imaginal discs arise from cells that are set aside late in embryogenesis and grow actively through much of larval life⁴¹. Their formation and growth are not blocked by JH, although JH may influence the rate of growth^{42,43}.

These 'classic' imaginal discs, though, seem to be derived structures⁴⁴, and may not be the proper type of imaginal primordia to compare with embryos. The ancestral condition for imaginal growth is thought to be similar to that seen for formation of the wing in the mealworm beetle, *Tenebrio molitor*⁴⁵. In the preterminal instars, the beetle larvae show no imaginal discs and the cells that will form the wing primordium simply secrete larval cuticle. Only in the last larval instar do these cells become columnar, detach from the overlying larval cuticle and begin their imaginal proliferation.

Figure 4 maps the pattern of wing growth onto a phylogenetic tree for the Holometabola. If we assume that the onset of imaginal growth in the last larval stage is the ancestral condition, then early-developing wing discs evolved independently at least six times. In contrast, if we assume that early-developing wing discs were

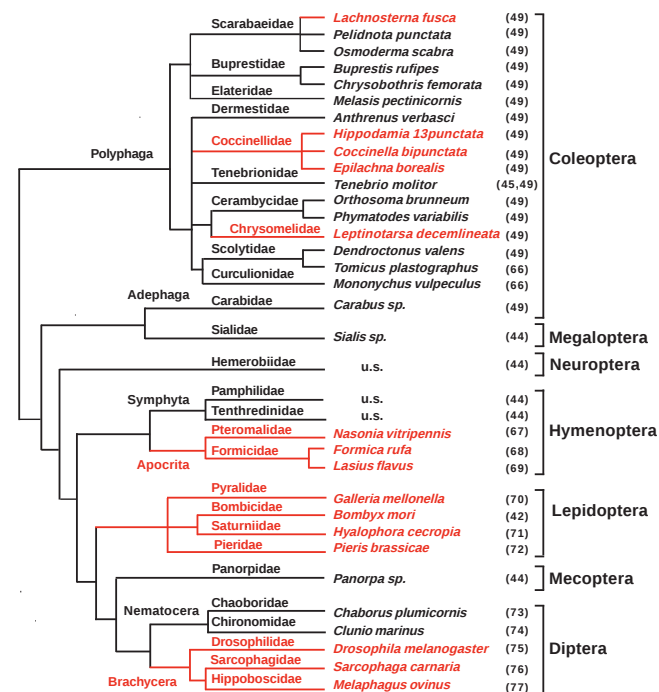


Figure 4 Relationship of the phylogeny of the Holometabola to the pattern of imaginal disc formation. For species in black, the proliferation of the wing primordium is delayed until the final larval instar. For species in red the wing-disc primordium is set aside from the general larval epidermis before the last larval stage and forms an invaginated imaginal disc that can grow more or less independently of the larval moult cycles. The red lines show where such imaginal discs would arise if the ancestral condition was to delay primordium formation until the last larval stage. They would have had to arise at least three times in the Coleoptera and at least once in the Diptera, Hymenoptera and Lepidoptera. SP, genus and species not determined. u.s., unidentified species. Reference numbers for wing-disc development are shown in parentheses. The phylogeny of the holometabolous orders is from ref. 64; that for the beetles is from ref. 65.

ancestral, then this capacity would have been lost at least ten times. Moreover, it would have been lost in the most primitive orders, Neuroptera (lacewings and antlions), Mecoptera (scorpionflies) and Megaloptera (alderflies), and also in the more basal members of the Diptera (the Nematocera: midges and mosquitoes) and the Hymenoptera (the Symphyta: sawflies) but preserved in the more derived groups. We think that the latter model is unlikely and accept the proposal¹⁰ that the ancestral holometabolous larva postponed their imaginal growth until the last larval stage.

In caterpillars of *M. sexta*, the wing primordia show a derived pattern of growth, but the primordia for the adult eyes show an ancestral growth pattern, in that they form only in the final larval instar^{46,47}. Studies both *in vivo* and *in vitro* show that JH directly inhibits eye primordium formation, whereas the premature removal of JH, even in preterminal larval instars, results in its precocious formation (D. Champlin, L.M.R. & J.W.T., unpublished results). Importantly, this action of JH is independent of the ecdysteroids.

We think that the control of the formation of the eye primordium represents a general model for the ancestral pattern of control over imaginal growth. JH tonically suppresses primordium formation throughout the preterminal instars, but its decline in the final instar removes the inhibition and allows rapid growth to begin. For the subsequent evolution of classic imaginal discs, the appropriate regions of the epidermis would have to escape this JH-mediated suppression so that they could form and proliferate despite the high JH titres that maintain the larval character of the rest of the animal. The mechanism(s) by which this was accomplished has not been determined but it could have involved the local loss of JH receptors or the acquisition of high levels of enzymes that locally inactivate JH. Consistent with the latter possibility, the wing discs of the wax moth *Galleria mellonella* express high levels of JH esterase activity⁴⁸ early in the last larval stage, which may facilitate their growth in an environment that still has circulating JH. Thus, the insensitivity of classic imaginal discs to JH is not surprising and, indeed, was a prerequisite to allowing imaginal growth throughout larval life and thereby shortening the life cycle.

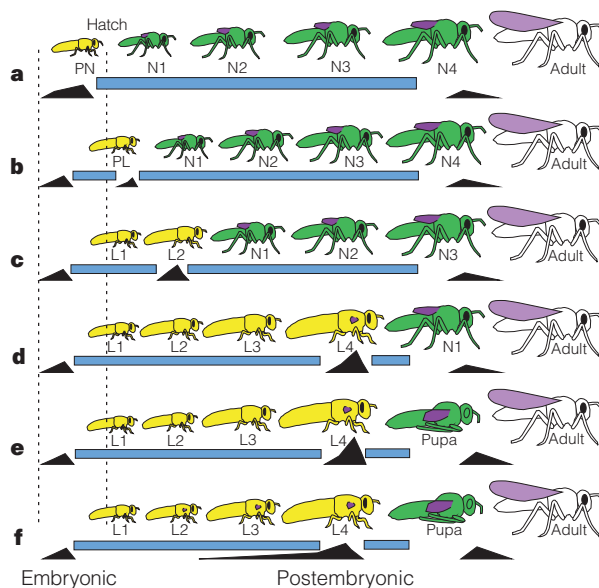


Figure 5 Possible steps in the transition from a hemimetabolous to a holometabolous life history and their relationship to timing of JH production (blue bar) and to growth directed towards the imaginal form (black triangles). The pronymphal (PN), protolarval (PL) and larval (L) instars are in yellow, and the nymphal (N) and pupal stages are in green; wing buds, wing imaginal discs, and wings are in purple. See text for explanation.

The main benefits ascribed to metamorphosis are the ability of larvae and adults to utilize different habitats and food resources, and the evolution of rapid life cycles. The selective pressures that initially gave rise to complete metamorphosis probably had to do with the former benefit of separating the resources used for the growth of the immature form from those needed for reproduction of the adult. After complete metamorphosis was established, the ability of imaginal discs to form and grow early in larval life then provided a mechanism that would allow the compression of life cycles¹⁰.

Evolution and wing primordia

The pattern of wing growth also has implications for the relationship of the pronymph to the larva. It is generally considered that the wing primordia grow as evaginated wing buds in hemimetabolous nymphs and as invaginated wing discs in holometabolous larvae. In hemimetabolous insects, the wing buds typically appear in the first or second nymphal instar and increase in size with subsequent nymphal moults. The appearance of wing buds in early nymphal instars is also evident in fossil hemimetabolous and ametabolous insects², so it seems to be a ubiquitous feature of both extinct and modern hemimetabolous insects. If the larva was derived from the nymph, then we have to conclude that this ability to form and grow wing primordia in preterminal nymphal stages was lost in the ancestor of the Holometabola but was then reacquired after metamorphosis evolved¹⁰. However, if the pronymph became the larva, then larvae would be expected to lack wing primordia, as all described pronymphs lack these structures. The wing primordia would form at the end of larval growth when the nymphal (pupal) stage was finally achieved. When they formed in the last larval instar, they might still have formed as evaginating structures, as is evident in some present-day larvae that have the ancestral pattern of wing formation⁴⁹. Invagination probably became the preferred mode of formation for these primordia because there is more space in the body cavity for the growth of a large structure like a wing disc. Invagination would be essential for wing primordium growth to be shifted into the early larval instars.

Evolution of metamorphosis

Our hypothesis for the evolution of metamorphosis is based on the premise that basal insects actually have three distinct life forms: pronymph, nymph and adult. We propose that these are directly comparable to the larval, pupal and adult stages of the Holometabola. In most hemimetabolous orders, with the possible exception of mayflies, the pronymph is a non-feeding stage (Fig. 5a). The ability of this stage to feed would seem to be an essential preadaptation for it to evolve into the larva. One hint as to how this might have occurred is provided by the embryos of modern-day Lepidoptera. During dorsal closure, only part of the yolk mass is enclosed by the developing embryo. Later, the developing caterpillar feeds on this extraembryonic yolk while still within the egg. If the embryos of the ancestor to the Holometabola also failed to enclose their yolk completely during dorsal closure, there would have been a selective advantage to modify the pronymph so that it could feed while still in the egg and thereby utilize this store of extraembryonic yolk. We will call such an animal a 'protolarva'.

The females of the Holometabola ancestor may have deposited their eggs in protected places, such as in the soil or under bark, thereby rendering them less vulnerable to predation. This behaviour would have selected for adaptations in the protolarva that allowed it to burrow efficiently out from these hiding places. As a feeding protolarva could also utilize food items that it encountered in this novel habitat (Fig. 5b), it could exploit resources that might not be available to either the nymph or the adult. If these resources were abundant, there would be a selective benefit to maintain this form into the next instar (Fig. 5c). We now consider this to be a true larval stage. Initially, postembryonic growth would be split between the larval and nymphal stages. As the nymph potentially competes with

the adult for food, selection for undergoing all growth in the larval form would completely separate the resources used for growth from those used for reproduction (Fig. 5d). This would reduce the nymphal stage to a single instar that no longer needed to feed but served as the transition between larva and adult; it became the pupa (Fig. 5e). It is interesting that at least one other hemimetabolous group, the thrips (Thysanoptera), may have independently attempted this transition. They typically have two 'larval' instars followed by two non-feeding 'nymphal' instars before becoming adults.

From an endocrine perspective, we think that the shift from a pronymph to a protolarva involved a heterochronic shift in embryonic JH secretion. The earlier appearance of JH would have suppressed some aspects of embryonic growth, and its presence during the formation of the pronymph would have caused the precocious maturation of the embryonic tissues. The latter action would be necessary to transform the pronymph from a transitional developmental stage into a protolarva with functional organ and tissue systems (Fig. 5b). The continuing presence of JH then maintained the form of the protolarva/larva (Fig. 5b, c). For the protolarva/larva to progress subsequently to the nymph stage, JH would have to disappear to allow the morphogenetic growth needed for the new nymphal structures, but then JH would return to support their maturation. Once the nymphal form was achieved, it would also be stable as long as JH was present. As the time spent as a protolarva/larva was extended, the JH-free period was progressively delayed until it was finally pushed to the end of the growth period (Fig. 5d). In this view, the anomalous reappearance of JH during the larval-pupal transition is directly related to the ancestral need for JH during the pronymph-nymph transition.

As the larva and nymph diverged, the amount of proliferation and tissue reorganization that had to occur after the JH decline became progressively greater. Also, as discussed above, the ability of selected tissues to become JH-independent allowed the development of imaginal discs and the marked reduction in the duration of life cycles (Fig. 5f).

Implications

The ancestral goal of embryonic development was to produce an individual that, with minor exceptions like wings and genitalia, was basically a miniature copy of the adult. During the evolution of metamorphosis, a heterochronic shift in the time of appearance of JH during embryogenesis may have interrupted this ancestral growth trajectory. How would such an interruption have affected the genetic cascades that are used for patterning the body axis and the limbs^{50,51}? In the case of an appendage such as the leg, one possibility is that the early JH secretion interrupted the progressive patterning of the limb bud and then stabilized it at an intermediate state. This intermediate set of patterning information would then be the basis for establishing the unique morphology of the larval leg. With the disappearance of JH in the final larval instar, this intermediate condition could no longer be maintained and the resumption of patterning interactions would provoke the growth of imaginal primordia, eventually resulting in the patterning of a typical adult limb.

Exogenous JH has striking effects on the embryogenesis of ametabolous insects⁵² but only minimal effects on their postembryonic development⁵³. In progressively more advanced groups, the effects of JH on embryogenesis become less marked while its postembryonic effects become more so. This trend suggests that the ancestral developmental role of JH was to control aspects of embryogenesis. The emergence of JH as a major postembryonic hormone accompanied the shift of some phases of embryonic growth (first of the genitalia and wings) into postembryonic life. The prospect of JH's originally being involved in embryonic development is intriguing because of the structural similarity of the JHs with the retinoids that regulate aspects of embryogenesis in vertebrates⁵⁴. In both groups of animal, these types of compound

may be ancient regulators of embryonic growth and development, but in the insects they have achieved a postembryonic, hormonal function as greater portions of embryonic development were deferred until the end of larval growth. □

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The unusual afterglow of the γ -ray burst of 26 March 1998 as evidence for a supernova connection

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Cosmic γ -ray bursts have now been firmly established as one of the most powerful phenomena in the Universe, releasing almost the rest-mass energy of a neutron star within the space of a few seconds (ref. 1). The two most popular models to explain γ -ray bursts are the coalescence of two compact objects such as neutron stars or black holes, or the catastrophic collapse of a massive star in a very energetic supernova-like explosion^{2,3}. Here we show that,

about three weeks after the γ -ray burst of 26 March 1998, the transient optical source associated with the burst brightened to about 60 times the expected flux, based upon an extrapolation of the initial light curve. Moreover, the spectrum changed dramatically, with the colour becoming extremely red. We argue that the new source is an underlying supernova. If our hypothesis is true then this provides evidence linking cosmologically located γ -ray bursts with deaths of massive stars.

Following the localization⁴ of the γ -ray burst (GRB) of 26 March 1998 (GRB980326) by the X-ray satellite BeppoSAX, Groot *et al.*⁵ quickly identified the optical afterglow. Our optical follow-up programme began at the Keck Observatory, approximately 10 h after the burst. A log of these observations is given in Table 1. In Fig. 1 we present our R-band photometry, along with values reported by other workers. Considering only reported data taken within the first month of the burst^{5,6}, we find a characteristic power-law decay in the flux versus time, followed by an apparent flattening. The usual interpretation is that the decaying flux is the afterglow emission, while the constant flux is due to the host galaxy.

But to our surprise, our more recent observations (first performed nine months after the GRB event) showed no galaxy at the position of the optical transient (OT). We estimate a 2σ upper limit to the R-band magnitude of $R > 27.3$ mag (see Table 1). This is almost a factor of 10 less flux than that reported from our 17 April detection. A secure conclusion is that the presumed host galaxy of GRB980326, assuming that the GRB was coincident with the host (as appears to be the case for all other well-studied GRBs to date), is fainter than $R \approx 27$ mag. This conclusion is not alarming as such faint (or fainter) galaxies are indeed expected from studies^{7,8} of the properties of cosmological GRB host galaxies.

Having established that the host galaxy of GRB980326 is faint, we are forced to conclude that the OT did not continue the rapid decay it exhibited initially. Instead, we find three phases of the light curve (Fig. 1): a steeply declining initial phase (at times since the burst of $t \lesssim 5$ d), a subsequent rebrightening phase ($t \approx 3$ –4 weeks) and

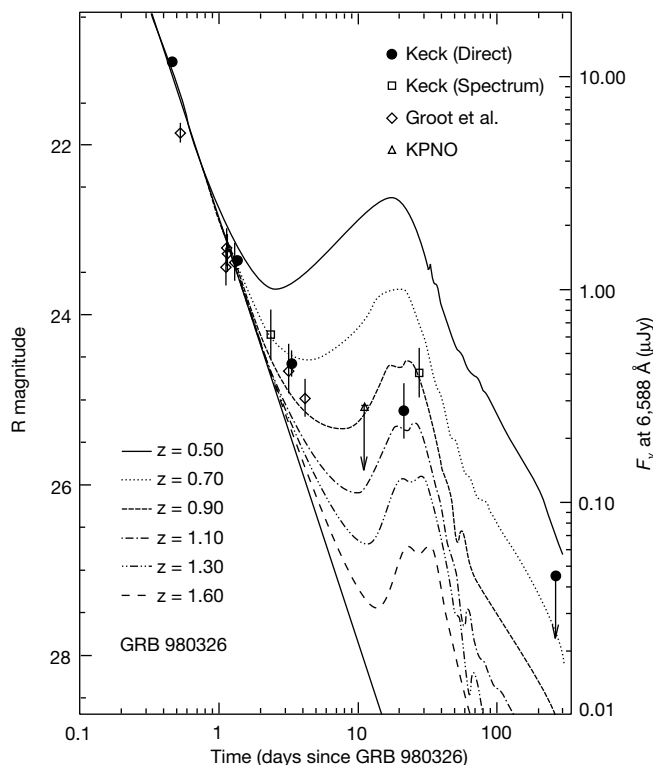


Figure 1 Analysis of the R-band light curve of the afterglow of GRB980326. Overlaid is a power-law afterglow decline summed with a bright supernova light curve at different redshifts. (Although we use as a template the multi-band light curve of SN1998bw (ref. 19), the bright supernova potentially associated with GRB980425, we emphasize that the exact light-curve shapes of a supernova accompanying a GRB is not known *a priori*.) The 'GRB + supernova' model at a redshift of about unity provides an adequate description of the data. From ref. 28 we estimate the Galactic extinction in the direction of the optical transient (Galactic longitude and latitude, 242.36° and 13.04° , respectively) to be $E(B - V) = 0.08$. Thus, assuming the average Galactic extinction curve ($R_V = 3.1$), the extinction measure is $A_B = 0.22$, $A_V = 0.16$ mag. Plotted are the extinction corrected magnitudes (see Table 1) and fluxes of the transient converted to the standard flux zero-point of the Cousins R filter (from ref. 26). In addition to our data, we include photometric detections from Groot *et al.*⁵ and an upper limit from Valdes *et al.*⁶ (KPNO). The GRB transient flux dominates at early times, but with a power-law decline slope $\alpha = -2$ (straight solid line). The supernova light-curve template was constructed by spline-fitting the broadband spectrum measured by Galama *et al.*¹⁹ of the bright supernova 1998bw at various epochs (augmented with late-time observations of SN1998bw by McKenzie and Schaefer²⁹) and transforming back to the rest frame of SN1998bw ($z = 0.0088$). As discussed in the text, we expect the rest-frame ultraviolet emission (below $3,900 \text{ \AA}$) to be suppressed due to absorption by resonance lines. We assume that the ultraviolet flux of the accompanying supernova is represented as $f_\nu \propto \nu^{-3}$. Theoretical light curves are then constructed by red-shifting the template to various redshifts and determining the flux in the R band (observer frame) by interpolating (or extrapolating, for $z \geq 1$). The flux normalization of the redshifted SN1998bw curves are independent of the Hubble constant, but are dependent upon the value of Ω_0 and Λ_0 (here we show the curves for $\Omega_0 = 0.2$ and $\Lambda_0 = 0$). Beyond $z \approx 1.3$, the observed R-band corresponds to rest-frame $\lambda \lesssim 2,900 \text{ \AA}$. As stated in the text, the spectrum in this range has been modelled along simple lines. Qualitatively, the peak flux derived from the supernova model as a function of redshift and shown here agrees with the theoretical peak flux-redshift relation for type I supernovae³⁰. This suggests that our adopted model for the ultraviolet spectrum is reasonable.

finally, a phase in which the source appears to have faded away to an undetectable level by the time of our next observation (9 months after the burst).

In previously studied bursts, the optical afterglow emission has been modelled by a power-law function, $\text{flux} \propto t^\alpha$; here α is the power-law index. In some bursts, at early times (t less than a day or so), significant deviations have been seen; for example, GRB970508 (ref. 9). At late times, in some bursts, deviations manifest as the steepening (that is, α becoming smaller) of light curves; for example, GRB990123 and GRB990510.

It is against this backdrop of the observed afterglow phenomenology that we now analyse the light curve in Fig. 1. The declining phase cannot be fitted by a simple power law ($\chi^2 = 72$ for 9 degrees of freedom). From Fig. 1 it is clear that the flux had already started flattening by day 3. Restricting the analysis to the first two days, we obtain $\alpha = -2.0 \pm 0.1$, consistent with a previous analysis⁵.

Such power-law decays are usually interpreted as arising from electrons shocked by the explosive debris sweeping up the ambient medium. Assuming that the electrons behind the shock are accelerated to a power-law differential energy distribution with index $-p$, on general grounds¹⁰ we expect that the afterglow flux $f_\nu(t)$ is proportional to $t^\alpha \nu^\beta$; here $f_\nu(t)$ is the flux at frequency ν and time t . The value of α and β depend on p , the geometry of the emitting surface¹¹ (spherical versus collimated) and the radial distribution of the medium around the burst¹².

From our spectroscopic observations of 29 March (Fig. 2), we find $\beta = -0.8 \pm 0.4$. This combination of (α, β) is similar to the $(\alpha = -2.05 \pm 0.04, \beta = -1.20 \pm 0.25)$ seen in GRB980519 (ref. 13), and can be reasonably interpreted¹⁴ as arising from a standard $p \approx 2.2$ shock with a jet-like emitting surface. Alternatively, the emission could arise in a $p \approx 3$ shock propagating in a circumburst medium¹² whose density falls as the inverse square of the distance from the explosion site.

Figure 2 The spectra of the transient on March 29.27 and April 23.25 1998 UT. The two spectra are shown at three different spectral resolutions. Panels **a** and **c** show the spectra at the two epochs at the full spectral resolution (see below for details); panels **b** and **d** show the same two spectra but binned in groups of 51 channels (solid line) and smoothed (dashed line). Panel **e** is the spectrum of the sky. The best-fit power-law models ($f_\nu \propto \nu^\beta$) to the binned spectra are shown by dashed lines; the fits were restricted to the wavelength range 4,500–8,500 Å. The scatter of individual channel values within each bin was used to assign relative weights to the median fluxes in each bin when performing the fits. We obtain $\beta = -0.8 \pm 0.4$ (March 29.27) and $\beta = -2.8 \pm 0.3$ (April 23.25). The derived power-law indices include the correction of Galactic extinction. The observations were obtained on LRIS²⁵ at the Keck II 10-m telescope with the 300 lines mm⁻¹ grating blazed at $\sim 5,000$ Å. On 29 March we employed a 1.0-arcsec slit and on 23 April a 1.5-arcsec slit. The effective wavelength coverage was $\lambda \sim 4,000$ –9,000 Å, and the instrumental resolution was ~ 12 Å (29 March) and 16 Å (23 April). Two (three) exposures of 1,800-s each were obtained. The estimated uncertainty of the flux zero point is $\sim 20\%$ on 29 March (calibrator Feige34³¹) but only 10% on 23 April (calibrator HD84937³²). On both epochs, exposures of arc lamps were used for primary wavelength calibration. Night sky lines were used to correct for calibration changes due to flexure. In both cases, slit position angles were close to the parallactic angles. Thus the differential slit losses were negligible. The spectra shown were convolved with a gaussian with $\sigma = 5$ Å (that is, less than the instrumental resolution) and rebinned to a common 5-Å sampling. None of the apparent features in the spectra are real: all are due to imperfect sky subtraction noise. A sky spectrum from the 23 April observation, extracted in the same aperture, is shown for the comparison. These spectra are shown before the correction for the Galactic foreground extinction.

We now discuss the bright source seen in the rebrightening phase. This source is ~ 60 times brighter than that extrapolated from the rapidly declining afterglow. Given that such a magnitude of excess at late times has not been reported before, it is important to review the crucial observations of mid-April. First, the rebrightened source is coincident with the position of the OT in the image of 27 March to within the expected astrometric error. It is consistently detected in three separate frames taken on 17 April. In the summed image, the source is detected at 4.6σ (chance probability of 2×10^{-6}) on 17 April and all other objects in the field at this level are reliably detected in our deeper 18 December image. Next, the source is clearly detected (spectroscopically) on 23 April (Fig. 2) at the same position as that of the OT. The inferred spectrophotometric R-band magnitude is plotted as open squares on the light curve (Fig. 1). Thus we conclude that there was indeed a source at the position of the OT which brightened three weeks after the burst and subsequently faded to undetectable levels. We now investigate possible explanations for this source.

The simplest picture is that the afterglow rebrightened. Piro *et al.*¹⁵ have recently suggested that the doubling of the X-ray flux of GRB970508 three days after the GRB event arises from the relativistic shell running into a dense gas cloud. Such an explanation for the GRB980326 light curve would require a large dense region, with a size comparable to the timescale of rebrightening, about $\Gamma \times 10$ light days (~ 0.01 pc), and located at a distance ~ 0.1 pc from the explosion site. Here, Γ is the bulk Lorentz factor of the shock and is expected to be of the order of unity three weeks after the burst. Panaitescu *et al.*¹⁶ suggest that the rebrightening of GRB970508 may be due to delayed energy injection by the extremely long-lived central engine that produced the GRB. Alternatively, the delayed energy could come from the spindown of a newly formed millisecond pulsar through magnetic dipole radiation¹⁷. For all these models, however, the expected spectrum would be the typical

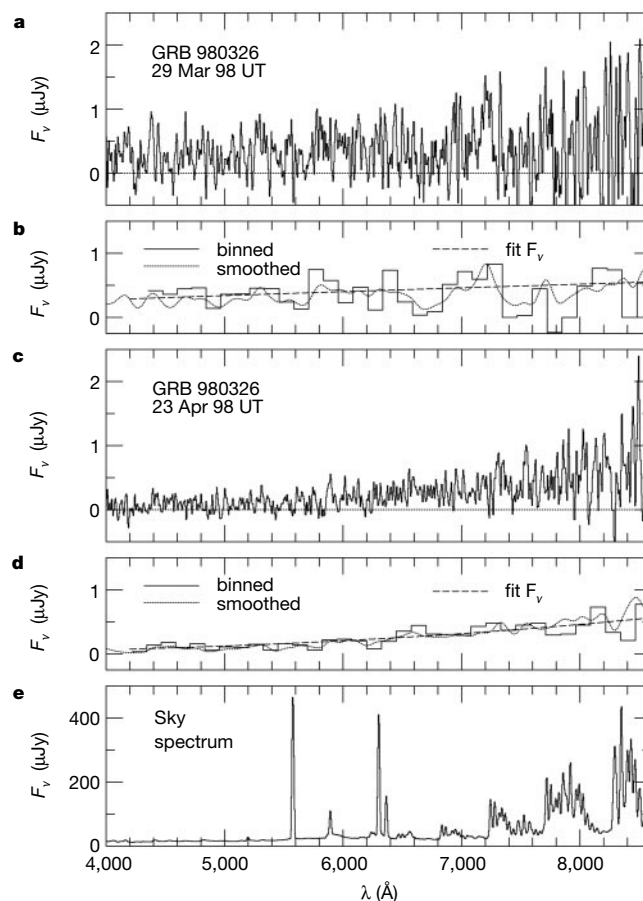


Table 1 Keck II optical observations of GRB980326

Date* (UT)	Band/ grating	Int. time (s)	Seeing (FWHM)	Magnitude†	Observers
Mar 27.35	R	240	0.74"	21.25 ± 0.03	A.V.F., D.C.L., A.G.R.
Mar 28.25	R	240	0.66"	23.58 ± 0.07	H.S., A.D., D.S., S.A. Stanford
Mar 29.27	300	3,600		24.45 ± 0.3‡	H.S., A.D., D.S., S.A. Stanford
Mar 30.24	R	900	0.93"	24.80 ± 0.15	S.P., B.G., R.K., I.M.H.
Apr 17.25	R	900	0.82"	25.34 ± 0.33	P.C., J.P.B.
Apr 23.25	300	5,400		24.9 ± 0.3‡	S.G.D., S.C.O.
Dec 18.50	R	2,400	0.74"	>27.3	S.R.K., J.S.B., M.H.v. Kerkwijk
Dec 18.54	I	2,100	0.74"	>25.3	S.R.K., J.S.B., M.H.v. Kerkwijk
Mar 24	I	5,450	0.80"	>26.6	S.R.K., J.S.B.

The epoch of GRB980326 is March 26.888, 1998 (ref. 4). We used the Keck II 10-m Telescope 2,048 × 2,048 pixel CCD (charged coupled device) Low-Resolution Imaging Spectrometer²⁵ (LRIS) for imaging and spectroscopy of the GRB field.

* Mean epoch of the image. The year is 1998 for all images except for that on 24 March for which it is 1999.

† Photometric calibration. The absolute zero-point of the R (effective wavelength²⁶ of $\lambda_{eff} \approx 6,588 \text{ \AA}$) and I bands ($\lambda_{eff} \approx 8,060 \text{ \AA}$) were calibrated to the standard Cousins bandpass using standard stars in the field SA98 (ref. 27) and assuming the standard atmospheric correction on Mauna Kea (0.1 mag per unit airmass, respectively). The estimated statistical error on the absolute zero-point is 0.01 mag. We estimate the systematic error (due to lack of inclusion of colour term) to be <0.1 mag. We propagated all photometry to the absolute zero-point derived in the first epoch of observation using eight "secondary" stars which were detected with high signal-to-noise ratio, unsaturated, near to the transient, and common to every epoch; the typical uncertainty in the zero-point propagation is 0.01 mag. Thus any systematic error in our absolute zero-point will not affect the conclusions based on relative flux. The quoted uncertainties contain all known sources of error (aperture correction, and so on). The calibrated magnitudes of the secondary stars reported in Groot *et al.*⁵ agree to within the measurement errors.

‡ Spectrophotometric measurement. The flux in μJy is determined at 6,588 $\text{\AA}$, the central wavelength of the R_c band; the conversion to magnitude assumes 0 mag equal to 3,020 Jy (ref. 26). The spectrophotometric magnitudes are relative to a bright star that was on the slit (for which we have obtained independent photometry from our images). As we chose a slit position angle close to parallactic, the calibration serves to eliminate most of the systematics and calibration errors; that is, the spectrophotometric magnitudes were put on the direct CCD system, and are not based on the flux calibration of the spectra (which do, nevertheless, agree to 20%).

synchrotron spectrum, flux $f_\nu \propto \nu^{-1}$ (or flatter). The very red spectrum of 21 April (Fig. 2) allows us to essentially rule out a synchrotron origin for the rebrightening phase of GRB980326.

Alternatively, as suggested by A. Loeb (personal communication), the GRB could have occurred in a dusty region and the afterglow would rebrighten as the dust is sublimated by the afterglow. However, the observed spectral evolution from a relatively blue spectrum (29 March) to red (23 April) moves in a direction opposite to that expected in this model. Last, a non-relativistic, thermal expanding envelope powered by radioactive decay could accompany the merger of a compact binary system¹⁸ that gives rise to the GRB and the afterglow: this envelope would radiate at luminosities comparable to that of a bright supernova, and should produce a red spectrum similar to that seen on April 23. However, the timescale for the peak emission is more than an order of magnitude shorter than the timescale for the rebrightening that we see with GRB980326.

We advance the hypothesis that the new source is due to an underlying supernova revealed only after the afterglow emission has vanished. Woosley and collaborators (see ref. 2 and references therein) have pioneered the "collapsar" model in which GRBs arise from the death of massive stars—stars which produce black-hole remnants rather than neutron stars. In this model, the iron core of a massive star collapses to a black hole, and releases up to a few times 10^{52} erg of kinetic energy. Some fraction of this energy is expected to emerge in the form of a jet with little entrained matter; bursts of γ -rays result from internal shocks in this jet. The remaining energy is absorbed by the star, causing it to explode and thereby produce a supernova.

Thus in this model, the total light curve has two distinct contributions: a power-law decaying afterglow component, and emission from the underlying supernova. In Fig. 1 we show the light curve expected in this model, and use the light curve of the well observed¹⁹ SN1998bw as a template for the supernova contribution. We find the R-band and I-band data to be consistent with a bright supernova at $z \approx 1$.

The very red spectrum of the source on 23 April finds a natural explanation in the supernova hypothesis. On theoretical² and phenomenological²⁰ grounds, we expect GRBs to arise from massive stars which have lost their hydrogen envelope, that is, type Ibc supernovae. At low redshifts, all type I supernovae are observed to exhibit a strong ultraviolet deficit relative to the blackbody fit to their spectra. This deficit is due to absorption by prominent atomic resonance lines starting below $\sim 3,900 \text{ \AA}$. Below $\lambda_c \approx 2,900 \text{ \AA}$, we expect to see very little flux. In the near-ultraviolet range (3,000–4,000 $\text{\AA}$), all spectra from type I supernovae have a red appearance. Approximating the flux by a power law ($f_\nu \propto \nu^\beta$), the ultraviolet power-law index (depending on the specific wavelength range chosen) is -3 or even smaller, as found in the prototypical type Ic SN1994I (ref. 21). Fitting the spectrum of Fig. 2 to a power law, we obtain $\beta = -2.8 \pm 0.3$. Such a red spectrum (negative β) requires that the observed spectrum correspond to the ultraviolet–blue region in the rest frame of the object. A smaller redshift would lead to a larger β . A larger redshift would substantially suppress the light in the observed R band (which covers the wavelength range 5,800–7,380 $\text{\AA}$). The detection in R band then provides an independent constraint (Fig. 1), $z \lesssim 1.6$.

We have used the light curve of SN1998bw because it is a very well studied type Ibc supernova with a possible association with GRB980425. We do not know *a priori* the precise spectrum and light curve of a supernova accompanying GRBs. In the collapsar model, the progenitor stars are expected to have no significant envelopes and thus the expected supernovae are of type Ibc. The general shape of the spectra of all type Ibc supernovae are expected to be the same, and are summarized above. Given the low signal-to-noise ratio of the 23 April spectrum and the expected line broadening due to high photospheric velocity we do not, as seems to be the case, expect to see any features in our spectrum.

Independently, from the absence of strong spectral breaks in our spectrum of the OT, we can firmly place the redshift of the OT at $\lesssim 2.3$. This constraint is consistent with our deduction that $z \lesssim 1.6$ (see above). Thus from a variety of accounts we find a plausible redshift of around unity for GRB980326. Such a redshift is not entirely unexpected. Indeed, we note that five out of eight spectroscopically confirmed redshifts of GRBs lie in the range $0.7 < z < 1.1$.

The direct evidence for an accompanying supernova can be seen in the light curve at timescales comparable to the time taken for supernovae to peak, $\sim 20(1+z)$ days. However, in our opinion three conditions must be satisfied in order to see the underlying supernova even when one was present. (1) The GRB afterglow should decline rapidly, otherwise the supernova will remain overpowered by the afterglow for all epochs. (2) Given the strong ultraviolet absorption (discussed above), only GRBs with redshift $z \lesssim 1.6$ have an observable supernova component in the optical band. (3) The host must be dimmer than the peak magnitude of the supernova ($M_V \approx -19.5$). The last requirement is not needed if the GRB can be resolved from the host (for example, by using the Hubble Space Telescope). Finally, one caveat is worth noting: the peak magnitudes of type Ibc supernovae are not constant (unlike those of type Ia), and can vary²² from -16 to 19.5 mag or even brighter²³. We have investigated the small sample of GRBs with adequate long-term follow up and conclude that perhaps only GRB980519 satisfies the first and the third observational conditions for supernova detection; the redshift of this GRB is unfortunately unknown.

If we accept the supernova interpretation for GRB980326, a long-duration (5 s) GRB, then it is only reasonable to posit that all other long-duration GRBs are also associated with supernovae. We suggest that sensitive observations be made—especially at longer wavelengths, to avoid the ultraviolet cutoff of supernovae—of GRBs satisfying the above three conditions. If our hypothesis is correct, then the light curves and the spectra of such GRBs would

show the behaviour shown in Figs 1 and 2 and discussed here. Indeed, motivated by this work, evidence for underlying supernovae in other GRBs is now being reported (GRB970228; ref. 24). *Note added in proof:* Galama, T. J. *et al.* (preprint astro-ph/9907264 at <http://xxx.lanl.gov>) (1999) have also recently reported supernova-like behaviour in the light curve and spectrum of GRB970228. □

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Disappearance of stellar debris disks around main-sequence stars after 400 million years

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Almost 5 billion years ago, the Sun formed in a local contraction of a cloud of molecular gas. A rotating disk of gas and dust is believed to have fed material onto the proto-Sun for the first few million years of its life, and to have formed the planets, comets and other Solar System objects. Similar disks, but with less mass, have been observed around a few main-sequence stars such as Vega¹. The dust particles orbiting stars like Vega will be removed on time-scales of the order of 1 Myr (Vega is about 350 Myr old), and therefore must be resupplied¹, at least for a time. But earlier surveys^{2,3} lacked the sensitivity to determine how many nearby stars have dust disks, and to investigate how long such disks survive. Here we report infrared observations indicating that most stars younger than 300 Myr have dust disks, while most older than 400 Myr do not: ninety per cent of the disks disappear when the star is between 300 and 400 Myr old. Several events that are related to the ‘clean up’ of debris in the early history of our Solar System have a similar timescale.

We used the ISOPHOT instrument⁴ on board the ISO satellite⁵, and measured the flux (at wavelengths of 25, 60 and 170 μm) of a sample of 84 nearby main-sequence stars of spectral types A, F, G and K. This sample excludes known multiple, variable or peculiar stars; that is, those for which the interpretation of the infrared emission would be ambiguous. The measurements were reduced using standard software (H.J.H. *et al.*, manuscript in preparation). We checked the quality of our measurements by comparing the measured flux densities with values predicted independently from visual magnitudes (B and V) using an empirical relation derived from IRAS measurements⁶. At 60 μm , the differences between prediction and measurement have a mean value $\mu = 4$ mJy and a dispersion $\sigma = 21$ mJy. The distribution has a pronounced tail

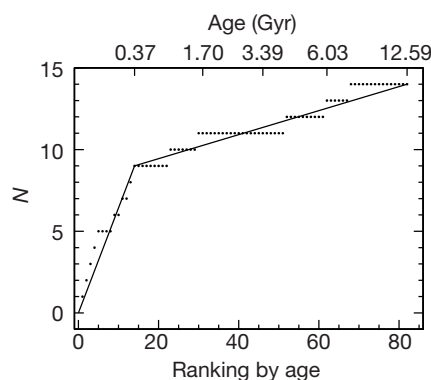


Figure 1 Cumulative distribution of the number of stars that have a disk. All stars are ranked by age. The x-axis shows the rank, and the y-axis the number of stars, N , up to that rank number that have a disk. Some ages are indicated at the top. The two straight lines fit the case when 60% of the stars with rank number lower than 15 (that is, younger than 380 Myr) have a disk and only 9% of the older stars have a disk.

Table 1 Stars younger than 1 Gyr arranged by age

HD	Name	Spectral type	Median age (Myr)	1 σ age limits (Myr)	Disk?
17925		K1V	80	40–150	Yes
30495	58 Eri	G3V	210	160–280	Yes
216956	α PsA	A3V	220	100–340	Yes
102647	β Leo	A3V	240	100–380	Yes
39060	β Pic	A3V	280*	0–520	Yes
20630	κ^1 Cet	G5V	300	180–500	
112185	ϵ UMa	A0p	300	280–1,200	
116842	80 UMa	A5V	320	280–500	
22049	ϵ Eri	K2V	330†	190–540	Yes
37394		K1V	340	270–430	
172167	α Lyr	A0V	350	310–390	Yes
74956	δ Vel	A1V	350	340–380	
95418	β UMa	A1V	360	330–400	Yes
38678	ζ Lep	A2V	370	230–490	Yes
103287	γ UMa	A0V	380	340–400	
15008	δ Hyi	A3V	450	400–500	
106591	δ UMa	A3V	480	420–540	
215789	ϵ Gru	A3V	540	510–580	
156026		K5V	630	440–910	
38392		K2V	650	360–1,100	
12311	α Hyi	F0V	810	780–870	
203280	α Cep	A7IV–V	890	810–980	

HD, identification number in the Henry Draper Catalogue.

* The age of β Pic is controversial; this value is large. See text.

† Age from rotation; calcium emission-line observations suggest²² 800 Myr.

containing stars for which more flux is measured than predicted: these are the stars with Vega-like infrared emission. We conclude that we have detected a disk when the measured flux is larger than the predicted flux by more than $\mu + 3\sigma = 67$ mJy. This gives 14 detections. In the remaining 70 stars, we detected the photosphere or an upper limit close to the photospheric flux. These stars have no disk, or at best a disk significantly fainter than the detected disks. The excellent correlation between predicted and measured flux in most stars confirms that the identification of the infrared source with the star is secure.

We determined an age for each star, and obtained good estimates for 81 of them. (Details of the determinations for 76 stars are reported elsewhere⁷.) For the remaining five stars— ϵ Eridani and four stars from another ISO paper⁸—we determined the age in the same way. The uncertainties in the age determinations are considerable (Table 1). We have taken a rather high value for the age of β Pictoris; a recent determination finds 20 Myr (ref. 9). Had we used that value, our conclusion (see below) would not have changed.

In Table 1 all 22 stars younger than 1 Gyr have been arranged according to increasing median age. The last column contains “yes” when a disk is detected. Below an age of 300 Myr, all stars have a disk. None of the stars in Table 1 older than 400 Myr have a disk, and of the 59 stars older than 1,000 Myr (and not in the table) only 6 have a disk. Future and better determinations of the ages of the stars will undoubtedly lead to a rearrangement of the precise order of the stars in Table 1, but we do not expect that this will change our main conclusion that the typical lifetime of a disk is 300–400 Myr. Figure 1 illustrates this conclusion in a different way (see Fig. 1 legend for details).

Two aspects of our results will be discussed here in more detail: the 300–400 Myr lifetime of the disks, and their disappearance.

We assume that the dust disks do not contain gas, and that the dust particles can move freely. Attempts to measure the gas content of the disk of β Pic¹⁰ justify this assumption. It follows that the dust particles will remain where they are for a time less than 1 Myr, a conclusion reached already in the discovery paper of the disk around Vega¹: radiation pressure will push the smaller particles out of the system, and the Poynting–Robertson effect will bring the larger ones in to where they are vaporized. For β Pic the survival time of the dust at 60 AU is only 4,000 years, and for α Piscis Austrini and Vega (α Lyr) it is 0.1–1 Myr (ref. 11).

If these dust particles are removed within 1 Myr, why do we see

disks with an age of a few hundred Myr? The answer must be: because there is a continuous supply of new dust. Plausible reservoirs of new particles are: collisions between large bodies, and evaporation of comets. Comets will evaporate only when they are within 1 AU or less from the star; the free dust particles will be pushed away to the outer region by radiation pressure. There is direct evidence for the existence of comets near β Pic and in a few other stars: narrow and variable components of the Ca II K-line in absorption suggest the infall of comet-like bodies¹².

We may estimate how much mass is needed for the replenishment. The mass of a disk as seen by ISO is of the order of 0.01 Earth masses^{13,14} ($0.01 M_{\oplus}$). If this disappears in 0.1 Myr and if it is replenished for 400 Myr, then the supply of dust must be $40 M_{\oplus}$ at least. A second, independent, argument leads to an even higher estimate: the collisional equilibrium distribution¹⁵, f , of the particle masses, m , equals $f(m) \propto m^{-1.83}$. If we assume that the largest particles have a size of $a = 100$ km, the total mass is 1.4×10^5 times what we see. If we take $40 M_{\oplus}$ of dust and add gas with a mass 100 times that of the dust, we obtain 0.01 solar masses, a typical value for a disk around a pre-main-sequence star. In other words, by removing its gas and by collecting the dust particles into large planetesimals, a pre-main-sequence disk will have evolved into the type of disk seen by ISO around main-sequence stars.

Are facts known about the history of the disk of our Solar System that correspond to what we know about the dust disks as seen by ISO? Our answer is “yes”. We consider initially the outer Solar System (that is, beyond the orbit of Neptune) because that corresponds to the region where the ISO dust disks are located.

First, model calculations of the formation of Pluto¹⁶, and of the time evolution of the inner Kuiper belt due to collisions¹⁷ and gravitational interaction with Neptune¹⁸, show that current observations are consistent with an early mass of the Kuiper belt of at least $30\text{--}50 M_{\oplus}$ and an initial erosion timescale of 400–800 Myr. Second, it is generally assumed¹⁹ that the Oort cloud of comets formed very shortly after the giant planets. These planets threw ice-covered planetesimals to the outermost regions of the Solar System. Third, the time and the duration of the so-called Late Heavy Bombardment in the inner Solar System coincide with the clean-up phase in the outer Solar System. It is possible, but not yet clear, that these two events are dynamically connected.

We propose that the stars mentioned in Table 1 are in the same phase as was our Solar System when it formed its planets and their satellites, its ring of asteroids, its Kuiper belt and its Oort cloud.

We note that not all debris disks decay after 400 Myr. In about 1 in 11 cases, the disk remains in existence over several Gyr. The disk around the 4.7-Gyr-old G2V star HD207129 is a good example¹⁴. Also, the 4-Gyr-old star ρ^1 Cancri harbours at least one planet²⁰ and a disk^{13,21}. It is currently unclear what causes these disks to live longer than most disks. □

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Rapid changes in the mechanism of ocean convection during the last glacial period

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High-amplitude, rapid climate fluctuations are common features of glacial times. The prominent changes in air temperature recorded in the Greenland ice cores^{1,2} are coherent with shifts in the magnitude of the northward heat flux carried by the North Atlantic surface ocean^{3,4}; changes in the ocean's thermohaline circulation are a key component in many explanations of this climate flickering⁵. Here we use stable-isotope and other sedimentological data to reveal specific oceanic reorganizations during these rapid climate-change events. Deep water was generated more or less continuously in the Nordic Seas during the latter part of the last glacial period (60 to 10 thousand years ago), but by two different mechanisms. The deep-water formation occurred by convection in the open ocean during warmer periods (interstadials). But during colder phases (stadials), a freshening of the surface ocean reduced or stopped open-ocean convection, and deep-water formation was instead driven by brine-release during sea-ice freezing. These shifting magnitudes and modes nested within the overall continuity of deep-water formation were probably important for the structuring and rapidity of the prevailing climate changes.

We studied two high-resolution mid-depth sediment cores, IMAGES MD95-2010 and ENAM93-21, from the Nordic Seas. Our main results come from core MD95-2010 (1,226 m depth; 66° 41.05' N, 04° 33.97' E) (Fig. 1), which we compare with results⁶

from core ENAM93-21 (1,020 m depth; 62° 44.3' N, 03° 59.92' W). A striking feature of these records is the high amplitude variability in benthic $\delta^{18}\text{O}$ (Fig. 2; see also Methods), which represents changes occurring at >1 km water depth. Almost identical large $\delta^{18}\text{O}$ variations on millennial timescales are recorded in the two cores. These strong fluctuations in deep water properties occur concomitantly with the rapid climatic changes (D–O cycles) recorded in the Greenland ice cores^{1,2}. Light isotopic events occur during the stadials (cold events), and heavy oxygen isotopic values occur during interstadial (warmer) events. This relationship is documented by the magnetic susceptibility (MS) record which shows detailed correlation with the Greenland ice cores (Fig. 1a, b), and between the cores (Fig. 2a; see Methods). Pronounced maxima (interstadial periods) and minima (stadial periods) in the MS record shift coherently with $\delta^{18}\text{O}$ fluctuations in GISP2. The stadials and interstadials are distinguished by the high content of ice rafted debris (IRD) within the stadials (high content of lithic grains during each stadial event) and by low IRD content during the interstadial events (Fig. 1d, e). Analogues to Heinrich layers, H1 to H6 (refs 3, 4), are identified by a high concentration of lithic grains, and by increased freshening of the surface waters as indicated by light $\delta^{18}\text{O}$

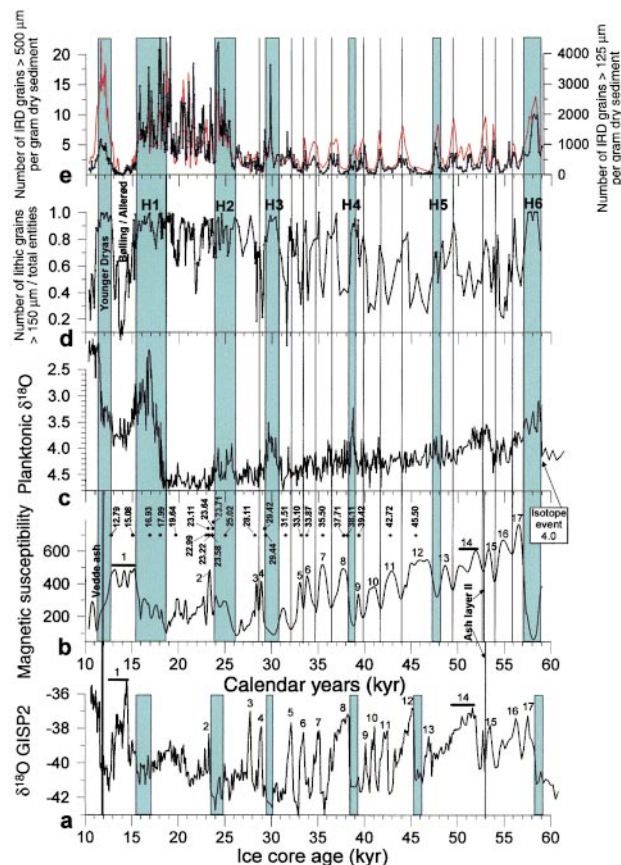


Figure 1 Stratigraphic data sets. Sediment core MD95-2010 versus the GISP2 ice core record. Comparisons of stadial and interstadial events documented from climate proxy data in MD95-2010 (**b–e**), and from the GISP2 ice core (**a**) are plotted on individual time scales (see Methods). Corresponding interstadial events (1–17) in the marine record and the ice core are indicated in **a** and **b**. **a**, GISP2 $\delta^{18}\text{O}$ record². **b**, Magnetic susceptibility (MS) record, with dated levels in MD95-2010 in calendar years indicated. **c**, $\delta^{18}\text{O}$ record of the planktonic species *N. pachyderma* sin. **d**, Ratio of lithic grains > 150 μm to the sum of lithic grains and foraminifera. **e**, Number of lithic grains per gram dry weight sediment. Size fraction > 500 μm shown in black, and size fraction > 150 μm in red. Coloured bars indicate placement of Heinrich-layer analogues, and vertical lines correspond to MS-minima intervals.

excursions in the planktonic record (Fig. 1c). The MS record and the number of lithic grains versus the total sum of lithic grains and foraminifera also correspond well (Fig. 1d).

To evaluate the aspects of the $\delta^{18}\text{O}$ variability which are due to the regional oceanographic factors we compare the benthic $\delta^{18}\text{O}$ record in MD95-2010 and ENAM93-21 with the Pacific Ocean core V19-30 (ref. 7), which reflects, in general, the global mean $\delta^{18}\text{O}$ change through the last glacial period (Fig. 2c). The main temperature shift in the deep Pacific took place during the main interglacial/glacial transitions and temperature changes in MIS3 and MIS2 are considered to be less than 1°C , reducing the light isotope peaks in the Nordic Seas relative to the global mean by 0.2‰ at most⁷. The $\delta^{18}\text{O}$ values from the Nordic Seas shift back and forth above and below the general global $\delta^{18}\text{O}$. Because the ice-volume effect is equally distributed in the oceans within the oceanic mixing time, the regional component of the $\delta^{18}\text{O}$ record is found by subtracting the $\delta^{18}\text{O}$ record in V19-30 from the $\delta^{18}\text{O}$ record in ENAM93-21 and MD95-2010. The resulting residual $\delta^{18}\text{O}$ records in MD95-2010 and ENAM93-21 (Fig. 2e) document the combination of gradients in temperature and the $\delta^{18}\text{O}$ of deep water between the deep Nordic Seas and the deep global ocean. The $\delta^{18}\text{O}$ values fluctuate consistently with the D–O cycles, with negative deviations (lighter $\delta^{18}\text{O}$

values in the Nordic Seas) during stadials and positive deviations during interstadials. The deviations are at times as much as 2‰ lighter (H1 and H6), and as much as 0.5‰ heavier than the $\delta^{18}\text{O}$ of V19-30, which represents a maximum $\Delta\delta^{18}\text{O}$ shift of 2.5‰ , from extreme minima to extreme maxima. In addition to H1 and H6, more than half of the stadial periods include benthic $\delta^{18}\text{O}$ amplitudes that are $1\text{--}1.5\text{‰}$ lighter. If temperature alone caused these changes, large temperature shifts of $5\text{--}11^\circ\text{C}$ would be required⁸. It is highly unlikely that the deep waters of the Nordic Seas changed temperature by these amounts. Water masses of such high temperatures would also not be dense enough to descend to depths in excess of 1 km, as they would need to attain the density of the water masses below this depth (previously formed by convection). Other mechanisms, therefore, which involve changes in the deep ocean $\delta^{18}\text{O}$ composition in the Nordic Seas, have to be invoked to explain the benthic $\delta^{18}\text{O}$ variations. Our conclusion is that these shifts from values much heavier than the global mean $\delta^{18}\text{O}$, to values much lighter, reflect shifts between modes of deep-water formation: open ocean convection was dominant during interstadials, and sea-ice freezing and brine formation were dominant during stadials, giving rise to the light isotope peaks.

Transfer of the light isotope (meltwater) signal from surface (planktonic) to depth (benthic) was rapid during the stadial/interstadial events (Fig. 2f, g). Lower surface water salinity during stadials are indicated by the light oxygen isotopic excursions in the planktonic foraminifer record during these intervals. This implies that open ocean convection ceased or was reduced during melting episodes owing to the buoyancy forcing of the added freshwater^{9,10}. There must therefore be alternative mechanisms to open ocean convection, which immediately brings the ^{18}O depleted surface water to intermediate depth ($>1,000\text{ m}$). The light isotope excursions in the stadials require a mechanism which creates large change in the $\delta^{18}\text{O}$ (δ_w) of the deep waters, and a rapid transit of the meltwater signal from surface to bottom. We suggest that this is best explained by brine formation. Isotopic fractionation during formation of sea ice is insignificant¹¹. Freezing of sea ice causes increased salinity S by salt rejection with almost no change in isotopic composition (Fig. 3a). The δ_w – S relationship (dashed line in Fig. 3a) shows that δ_w does not change noticeably over a salinity range of 1.3‰ due to sea-ice freezing which increases salinity/density with an isotopic fractionation close to zero (ref. 11). Hence, to explain light $\delta^{18}\text{O}$ values in the deep water we first need to induce freshwater which has a light isotopic signal somewhere at the ocean surface. This water is rapidly densified by sea-ice formation while retaining the light isotopic signature of the surface water. Figure 3b illustrates how surface ocean parameters (density, $\delta^{18}\text{O}$ and salinity) respond to these changes. In order to bring surface water to a density similar to that of the initial deep-water mass A_{DW} and maintain light isotopic values as indicated by point B, we introduce deep-water-formation processes in the Nordic Seas similar to those in the modern Weddell Sea (see Fig. 3a). Atmospheric cooling of surface water and strong prevailing winds from the ice sheet to the Nordic Seas are thought to produce sea ice on the shelves. Such conditions caused deep-water formation by brine injection as indicated by point C_{DW} in the T – S diagram (Fig. 3b). The salinity change ΔS required by the brine process to achieve the same density as in the initial deep water A_{DW} is about 1.5‰ . Owing to minor isotopic fractionation during sea-ice freezing, the resulting isotopic composition in the deep water, generated by brine formation, is indicated by point C'_{DW} in the upper δ_w – S diagram. By using different mixing lines (δ_w freshwater = -30‰) and sea level drop estimates (60 m), similar reasoning for the $\delta^{18}\text{O}$ variability in MIS3 indicates that sea-ice formation must increase salinity by about 0.8‰ to account for the freshwater input during stadials in MIS3. Salinity and $\delta^{18}\text{O}$ measurements in the Weddell Sea, a modern analogue for our brine model, show a ΔS gradient, from the upper homogeneous mixed layer to the deep ocean, of $0.7\text{--}1.3\text{‰}$

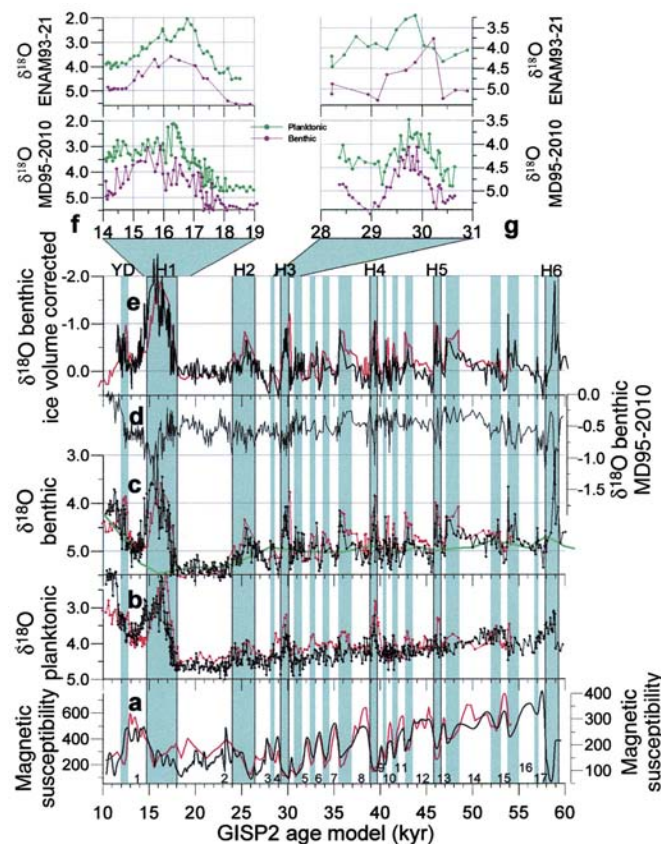


Figure 2 Climate proxy data in sediment cores MD95-2010 and ENAM93-21 (ref. 6). The data are plotted on the GISP 2 age scale (see Methods). Results from MD95-2010 and ENAM93-21 (a–e) are plotted in black and red, respectively. **a**, Magnetic susceptibility record. **b**, Planktonic $\delta^{18}\text{O}$ records (*N. pachyderma* sin.). **c**, Benthic $\delta^{18}\text{O}$ records (see Methods). Also shown is the benthic $\delta^{18}\text{O}$ record (green line) from the Pacific Ocean core V19-30 (ref. 7). The original $\delta^{18}\text{O}$ record for V19-30 is adjusted by $+0.4\text{‰}$ to account for the modern North Atlantic/Pacific sea water oxygen isotope gradient²⁸. **d**, Benthic $\delta^{13}\text{C}$ record in MD95-2010. **e**, Residual $\delta^{18}\text{O}$ records in MD95-2010 and ENAM93-21 after subtraction of the $\delta^{18}\text{O}$ record in V19-30 (see text). Coloured vertical bars correspond to MS-minima intervals, where H1–H6 are also indicated. **f**, **g**, Enlarged segments of the planktonic and benthic $\delta^{18}\text{O}$ records centred around H1 and H3, respectively.

without any significant changes in $\delta^{18}\text{O}$ (Fig. 3a). The results derived from the estimates above, from H1 ($\Delta S = 1.5\text{‰}$) and MIS3 ($\Delta S = 0.8\text{‰}$), are thus in close agreement with modern observations in the Weddell Sea.

The heavy oxygen-isotope values of the interstadials indicate water masses which cannot be significantly influenced by this type of deep-water formation, as they are isotopically heavier than other water masses of the global ocean^{12,13}. We interpret these intervals to reflect open ocean convection (deep water formation from surface waters unaffected by the addition of isotopically light water masses in the Nordic Seas). We conclude that common to all stadial–interstadial cycles (D–O and Heinrich-events) is a shift in the mode of overturning in the Nordic Seas, with normal deep-water formation (similar to the modern form, although less vigorous) in the warmer phases of the glacial, interrupted by a shift to deep-water formation dominated by brine-release during the cold phases with meltwater injection.

Rasmussen *et al.*⁶ argued that the variability in the benthic $\delta^{18}\text{O}$ (Fig. 2), and benthic foraminiferal fauna in ENAM93-21, indicated the presence of Glacial North Atlantic Intermediate Water (GNAIW) during stadial periods. This GNAIW was thought to have formed by convection south of the Greenland–Scotland ridge, instead of in the Nordic Seas, and would be a warmer water mass ($>0^\circ\text{C}$), which would explain some of the low benthic $\delta^{18}\text{O}$ in stadials. As discussed above, however, temperature changes are an improbable explanation for the high amplitude variability in $\delta^{18}\text{O}$. Nor would a convection site further south contain significantly more $\delta^{18}\text{O}$ depleted water than water masses in the north. On the

contrary, we would expect water masses in the south to be more saline, and consequently have higher $\delta^{18}\text{O}$ values (Fig. 3a). Also, if the bottom water in the stadials were generated from GNAIW, we would expect to see positive $\delta^{18}\text{O}$ values (ref. 19) connected with the negative $\delta^{18}\text{O}$ excursions. In general, however, the opposite is observed: peak negative $\delta^{18}\text{O}$ values are characterized by a drop in the $\delta^{18}\text{O}$ values (Fig. 2d).

Lehman *et al.*¹⁵ suggested that ^{18}O depleted freshwater may be sequestered at the ocean surface while the thermohaline circulation is in the collapsed state, and that the accumulated freshwater is later advected rapidly through the deep Atlantic as the circulation abruptly recovers. As a result, the most significant negative change in $\delta^{18}\text{O}$ of deep water should occur when the overturning circulation is re-established, and should not be associated with the coldest period and the time of greatest meltwater flux at the surface. Our data do not support this model. Instead, the benthic $\delta^{18}\text{O}$ minima are all connected with the cold periods and parallel the light $\delta^{18}\text{O}$ excursions in the planktonic isotope record, reflecting an almost instant transfer from surface to depth of the isotopically light surface water without any significant time lag (Fig. 2f, g). Spectral analysis of proxy data in MD95-210 and ENAM93-21 (see Supplementary Information) identifies the cycles with frequencies similar to those detected for Greenland ice cores^{16,17} showing spectral power at about 5 kyr, 2.3 kyr and 1.5 kyr. These results indicate that the shifts in the mode of deep-water formation are tied to the general climate variability.

Brine formation seems to be directly coupled to a cessation or strong reduction in open ocean convection, linked to periods of

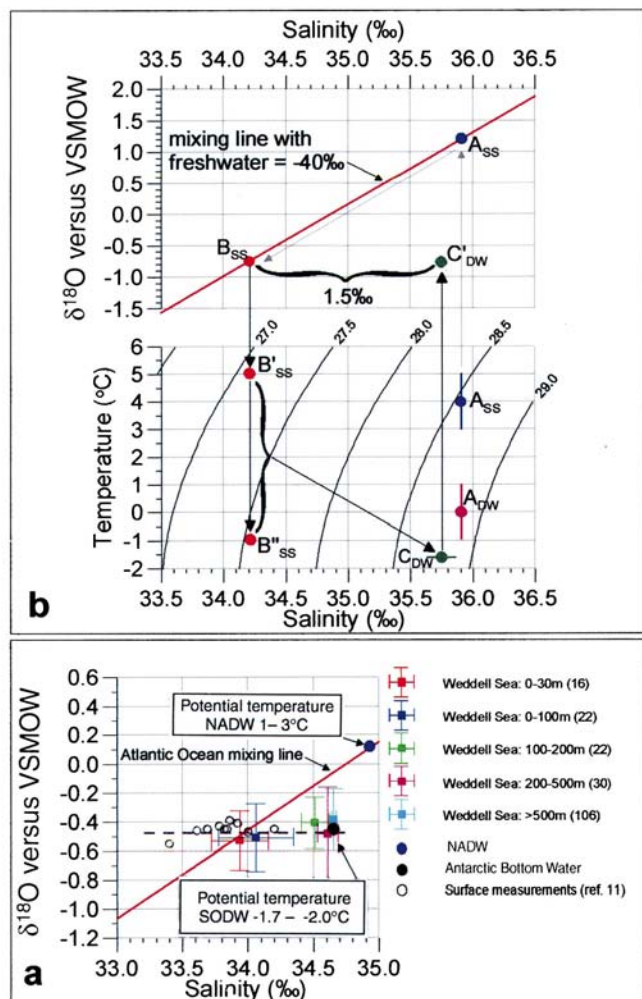


Figure 3 Relationships between salinity, $\delta^{18}\text{O}$ and sea-ice formation. **a**, Sea-ice formation causes Southern Ocean Deep Water (SODW) to fall off the main δ_w/S relationship for the surface water compared to North Atlantic Deep Water (NADW) which directly reflects the δ_w/S of surface water¹¹. Approximately 200 δ_w/S measurements from the Weddell Sea (S. Østerhus, personal communication) are included, shown as mean δ_w/S values with 1σ for specific depth. Additional δ_w/S surface water data from the Weddell Sea are also shown¹¹. **b**, Transformation of a sea surface water mass (A_{SS}) representative for the LGM (Last Glacial Maximum) surface water mass is influenced by meltwater with negative $\delta^{18}\text{O}$ during H1 and subsequently cooled and densified by sea ice formation to a density similar to deep water formed previously by open ocean convection (A_{DW}). In the lower T–S diagram, a typical LGM (Last Glacial Maximum) surface water mass A_{SS} is indicated, having a sea surface temperature of about 3–5 °C (ref. 29). Sea surface salinity is estimated by adding 1‰ to present-day salinity (34.91‰), which corresponds approximately to a sea level drop of 120 m (ref. 30). In the upper δ_w –S diagram, the same surface water mass A_{SS} is plotted by adding 1.1‰ (ice volume effect) to the modern δ_w value (=0.15‰). The salinity change as a result of adding freshwater to the surface water with a δ_w of –40‰, to obtain a 2‰ change in the planktonic $\delta^{18}\text{O}$ record, as observed within H1, is indicated by B_{SS} ($\Delta S = \text{salinity } A_{SS} - \text{salinity } B_{SS} = 1.7\text{‰}$). Projecting point B onto the T–S diagram gives the change in density from the initial surface water mass A_{SS} to the new freshwater influenced surface water mass B_{SS}. The resulting change in density, over a temperature range from –1 to 5 °C ($B'_{SS} - B_{SS}$), is 1 to 1.5 VSMOW, Vienna standard mean ocean water.

excess freshwater delivery to the surface ocean, and also to Northern Hemisphere cold intervals (stadials). We suggest that shelf areas, especially in the Barents Sea, and also along the coast of Greenland, Iceland and Norway, were potential source areas for intensive sea-ice formation. Such formation of sea ice would also be enhanced by less saline surface water masses, not only due to melting icebergs, but also because weak ocean conveyor circulation would reduce the salt flux to the surface of the North Atlantic region¹⁸. Pooling of fresh water at the ocean surface would act as a strong barrier against restarting the conveyor circulation; brine formation (and possibly also outflow of freshwater) may be important to re-establish open ocean convection¹⁹. Deep waters generated by brine formation sink to sill depths and below (core depths are deeper than sill). The estimated density would create overflows across the sills which would lead to a northward-compensating flow of surface water or near-surface water from the south. Estimates of salinity before, during, and after Heinrich-event 4 indicates that the surface water immediately to the south of the Nordic Seas was saltier than inside the Nordic Seas²⁰. The overflow could therefore potentially create a net northward salinity flux. Thus, brine formation may export salt out of the region, leaving the surface water fresher; yet brine formation and continuous deep-water formation may also enhance the inflow (of saltier surface water from the south) which is required to restart and run the open ocean convection. The creation of a net salinity flux to the north by brine formation may be an important mechanism, enabling rapid shifts to warm conditions, and hence the end of the stadials. □

Methods

Benthic stable isotope measurements

Cibicides wuellerstorfi is commonly used for benthic $\delta^{18}\text{O}$ -measurements; however, owing to limited abundance of *C. wuellerstorfi* during MIS2 and MIS3 we have used the benthic species *Cassidulinia teretis* for the $\delta^{18}\text{O}$ measurements. *C. teretis* is an epifaunal, or shallow infaunal form, feeding on organic debris at the surface or in the uppermost layer of the sediment²¹, and may therefore well reflect the $\delta^{18}\text{O}$ of the bottom water. *C. teretis* is considered to be calcifying in equilibrium with sea water²². In ENAM93-21 *Melonis barleeanum* is used for benthic $\delta^{18}\text{O}$ measurements⁶. These values have been corrected by 0.36‰ (refs 22, 23).

Age model and correlation to Greenland ice core records

In total, 28 AMS (accelerator mass spectrometry) ^{14}C samples were measured in MD95-2010 (Fig. 1b; see Supplementary Information for dates). All ages (>11 kyr) are transformed to calendar years using the equation of Bard *et al.*²⁴. Beyond the range of AMS ^{14}C ages the $\delta^{18}\text{O}$ record is correlated to the SPECMAP timescale²⁵. Our assumption is that the MS record and the IRD record in MD95-2010 represent interstadial-stadial variability which can be directly correlated to D-O events in GISP2 and GRIP ice core records (see also text). The synchrony and phasing between the marine record and the ice core record is difficult to evaluate in full detail. The following arguments add confidence to their correlation and suggest that stadial and interstadial periods in the marine environment are directly coupled to cold and warm periods in the ice cores, respectively. (1) We first tested the age model in MD95-2010 against the age model established for the GISP2 ice core (Fig. 1), which is based on annual layer counting down to about 50 kyr ago (ref. 26) and correlation to the VOSTOK ice core and SPECMAP beyond 50 kyr (ref. 27). Back to 60 kyr (calendar years) ago, corresponding stadial and interstadial periods in GISP2 and MD95-2010 agree, within the error or uncertainty of the ^{14}C -ages and the conversion from radiocarbon to calendar ages. The deviation in age between the two records is always less than 1.5 kyr (normally much less than 1 kyr) and is almost zero in the interval (10–40 k calendar years) where we may expect the AMS ^{14}C ages and conversion to calendar year to be most precise. We converted the age model in MD95-2010 into a GISP2 age model. The strategy we used was to correlate each minimum and maximum in the MS record with minima and maxima in the $\delta^{18}\text{O}$ record in GISP2. A simple linear regression between the two age models gave a linear relationship between the two independent age models of close to 1:1 (see Supplementary Information). (2) Two pronounced ash layers are recognised in the Greenland ice cores and in the Nordic Seas sediment cores. The Vedde ash is confined to the Younger Dryas cold period, and Ash layer II is identified, both in MD95-2010 and in the ice cores, within the stadial between interstadials 14 and 15. These two ash layers are thus direct evidence that these stadial periods are directly correlative between the ice cores and the marine cores. (3) Another independent tie-point for correlating the marine and ice core records comes from measurements of the geomagnetic field intensity in the marine cores (C. Kissel *et al.*, unpublished data; C. Laj *et al.*, manuscript in preparation). A well-defined low in the geomagnetic field corresponding to the Lachamp event, always falls within interstadial 10 in the marine record for the Nordic Seas and the North Atlantic. This change in the geomagnetic field directly affects the production of cosmogenic isotopes such as ^{10}Be , and therefore the ^{10}Be spike observed

from the GRIP ice core within interstadial 10 is strong evidence that these events are synchronous; also, at this point the correlation must be valid.

When converting the ages in MD95-2010 and ENAM93-21 on a common age scale, we have used the GISP2 age scale. We used the MS record for correlating MD95-2010 and ENAM93-21 (Fig. 2).

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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The melting curve of iron at the pressures of the Earth's core from *ab initio* calculations

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The solid inner core of the Earth and the liquid outer core consist mainly of iron¹ so that knowledge of the high-pressure thermodynamic properties of iron is important for understanding the Earth's deep interior. An accurate knowledge of the melting properties of iron is particularly important, as the temperature distribution in the core is relatively uncertain^{2–4} and a reliable estimate of the melting temperature of iron at the pressure of the inner-core boundary would put a much-needed constraint on core temperatures. Here we used *ab initio* methods to compute the free energies of both solid and liquid iron, and we argue that the resulting theoretical melting curve competes in accuracy with those obtained from high-pressure experiments. Our results give a melting temperature of iron of $\sim 6,700 \pm 600$ K at the pressure of the inner-core boundary, consistent with some of the experimental measurements. Our entirely *ab initio* methods should also be applicable to many other materials and problems.

The pressure P at the inner-core boundary (ICB) is 330 GPa (ref. 1), and the values for the melting temperature T_m of Fe are needed up to at least this pressure. Static compression measurements of T_m with the diamond anvil cell (DAC) have been made up to ~ 200 GPa, but even at lower pressures results for T_m disagree by several hundred kelvin (ref. 2). Recent DAC diffraction experiments³ demonstrate that in the range 60–80 GPa the solid phase from which Fe melts has a hexagonal close-packed ϵ -phase. Shock experiments are at present the only available method of T_m measurement at higher pressures, but their interpretation is not simple, and there is a scatter of at least 2,000 K in T_m at ICB pressures². Some experiments have been interpreted as indicating a solid phase different from the ϵ -phase at $P \geq 200$ GPa and $T \geq 4,000$ K, but the evidence is inconclusive²⁹. There have also been attempts to obtain the melting curve from parametrized atomistic models for the energetics of iron⁴, but the reliability of these models is uncertain.

Parameter-free *ab initio* techniques based on density functional theory (DFT) have recently made enormous strides⁵, and for many materials, including transition metals, reliance on parametrized models is now unnecessary. With *ab initio* molecular-dynamics methods⁶, this is true for both liquids and solids⁵. Solid iron in all its known crystal structures has been extensively studied with DFT. The accuracy of DFT depends on the approximation used for the electronic exchange-correlation energy, but with the generalized-gradient approximation⁷ (GGA) the experimentally observed properties of Fe are very accurately reproduced. These include the equilibrium lattice parameter, bulk modulus, magnetic moment⁸ and phonon frequencies⁹ of the body-centred cubic α -phase, the volume of the ϵ -phase as a function of pressure up to core pressures^{8,10,11}, and the low-temperature coexistence pressure of the α and ϵ phases^{8,10}. Extensive *ab initio* thermodynamics calculations^{12,13} for close-packed solid iron under Earth's core conditions have demonstrated impressive agreement with shock data and other experiments. Recently, we reported *ab initio* molecular dynamics simulations^{14–16} of liquid Fe and its alloys with sulphur and oxygen,

which we used to study the structure and viscosity of these liquids under core conditions.

Here we use the implementation of DFT known as the ‘projector augmented wave’^{17,18}. This is an all-electron technique in the sense that the valence wavefunctions are correctly represented everywhere in space (not ‘pseudized’ in the atomic-core regions as in pseudopotential methods), and resembles other all-electron methods such as FLAPW (full-potential linear augmented plane wave), used in previous work⁸ on solid Fe. It is also similar to the ultrasoft pseudopotential methods used in our earlier work, and, like them, it can be used for dynamical simulations. The present work was done with the Vienna Ab-initio Simulation Package (VASP) code¹⁹ which is extremely robust and stable for simulation of metals. Full technical details will be given elsewhere, but we note that a correct treatment of the $3p$ electrons (and to a much smaller extent the $3s$ electrons) is essential, since these respond significantly to their environment at the pressures of the Earth's core. These effects have been included as in our earlier work on the viscosity of iron¹⁴.

Since Fe melts from the ϵ -phase in the pressure range immediately above 60 GPa (ref. 3), we focus here on equilibrium between the ϵ and liquid phases. The condition for two phases to be in thermal equilibrium at a given temperature T and pressure P is that their Gibbs free energies $G(P, T)$ are equal. To determine T_m at any pressure, we calculate G for the solid and liquid phases as a function of T and determine where they are equal. In fact, we calculate the Helmholtz free energy $F(V, T)$ as a function of volume V , and hence obtain the pressure through the relation $P = -(\partial F/\partial V)_T$ and G through its definition $G = F + PV$.

Our calculations of F for solid and liquid Fe build on significant advances in *ab initio* free energy techniques^{20,21}. The free energy of the harmonic vibrating solid is conceptually simplest, and is given (per primitive cell) by the standard high-temperature formula: $F = F_{\text{perf}} + N_k^{-1} \sum_{\mathbf{k}s} \ln(\hbar\omega_{\mathbf{k}s}/k_B T)$ where F_{perf} is the free energy of the rigid perfect lattice and $\omega_{\mathbf{k}s}$ is the frequency of the phonon of branch s at wavevector $\mathbf{k}$. (Here $\hbar$ is Planck's constant divided by 2π , and k_B is Boltzmann's constant.) The sum goes over the N_k wavevectors in the Brillouin zone of the crystal and over the branches s . Note that F_{perf} contains an entropy contribution from thermal excitation of electrons: thermal electronic excitation is included throughout this work for both solid and liquid Fe phases.

To obtain melting properties with useful accuracy, free energies must be calculated with high precision, because the free-energy curves for liquid and solid cross at a shallow angle. It can readily be shown that to obtain T_m with a technical precision of 100 K, non-cancelling errors in G must be reduced below 10 meV. Errors in the rigid-lattice free energy F_{perf} due to basis-set incompleteness and

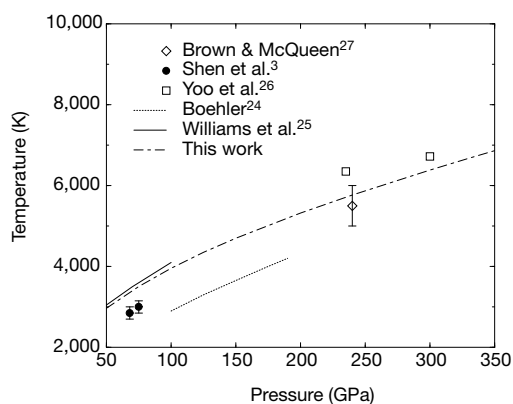


Figure 1 The *ab initio* melting curve of iron compared with experimental results. The dashed curve shows *ab initio* results; solid and dotted curves are interpolations of DAC measurements made by Williams *et al.*²⁵ and Boehler²⁴ respectively; the data points due to Shen *et al.*³ represent a lower bound rather than the melting curve itself; the squares and diamond with error bar are shock data from refs 26 and 27.

Brillouin-zone sampling are readily reduced to a few meV per atom. The frequencies $\omega_{\mathbf{k}}$ were obtained by diagonalizing the force-constant matrix; this matrix was calculated by our implementation of the small-displacement method described by Kresse *et al.*²². The difficulty in calculating the harmonic free energy is that $\omega_{\mathbf{k}}$ must be accurately converged for wavevectors $\mathbf{k}$ over the whole Brillouin zone. This requires that the free energy is fully converged with respect to the range of the force-constant matrix. To attain the necessary precision we used repeating cells containing 36 atoms, and to show that such cells suffice we went to cells of up to 150 atoms.

To calculate the liquid free energy and the anharmonic contribution to the solid free energy, we use the technique of 'thermodynamic integration', which yields the difference $\Delta F \equiv F - F_{\text{ref}}$ between the free energy of the *ab initio* system and that of a reference system F_{ref} . The basis of the technique^{21,23} is that ΔF is the work done on reversibly and isothermally switching from the reference total-energy function U_{ref} to the *ab initio* total energy U . This switching is done by passing through intermediate total-energy functions U_{λ} given by $U_{\lambda} = (1 - \lambda)U_{\text{ref}} + \lambda U$. It is a standard result that the work done is:

$$\Delta F = \int_0^1 d\lambda \langle U - U_{\text{ref}} \rangle_{\lambda} \quad (1)$$

where the thermal average $\langle U - U_{\text{ref}} \rangle$ is evaluated for the system governed by U_{λ} . The practical feasibility of calculating *ab initio* free energies of liquids and anharmonic solids depends on finding a reference system for which F_{ref} is readily calculable and the difference $U - U_{\text{ref}}$ is very small.

For the liquid, our threshold precision is only achievable because the *ab initio* energy U is extremely well reproduced by a model U_{ref} consisting of a sum of pair potentials $\phi(r)$, with $\phi(r)$ chosen to be an inverse-power repulsive potential: $\phi(r) = A/r^{\alpha}$. (Here r is the interatomic distance.) The parameters A and α are adjusted to minimize the strength of the fluctuations $U - U_{\text{ref}}$. We have demonstrated the excellence of this U_{ref} by doing *ab initio* dynamical simulations at 18 different thermodynamic states spanning the (P , T) range of interest, and we find that a single choice of A and α is almost equally good over the whole range. The free energy of the reference model is readily calculated for very large simulated systems, so that size errors are negligible. We then use thermodynamic integration to compute the difference ΔF giving the full *ab initio* free energy. The size errors on ΔF fall below 5 meV for *ab initio* systems of 125 atoms, as we have demonstrated using considerably larger systems; errors due to electronic k -point sampling are readily reduced to the same level. Most of our calculations were actually made using 67 atoms, but corrected for size and k -point errors by doing spot checks on 125 atoms. The inverse-power model is not a good enough reference system for the anharmonic solid, but we find that an extremely good reference system for this case can be obtained by suitably combining the inverse-power and harmonic *ab initio* total energies. Specifically, if U_{IP} is the total energy consisting of the sum of pair potentials $\phi(r)$, and U_{harm} is the harmonic part of the *ab initio* total energy, the reference system we use is $U_{\text{ref}} = c_1 U_{\text{IP}} + c_2 U_{\text{harm}}$, where c_1 and c_2 are appropriately chosen coefficients. We stress that the choice of reference system affects only the efficiency of the calculations, and not the final results.

Figure 1 compares our *ab initio* melting curve with experimental results from both DAC and shock measurements. For pressures $P < 200$ GPa covered by DAC, our curve lies $\sim 1,000$ K above the values of Boehler²⁴ and ~ 500 K above the more recent values of Shen *et al.*³ (who stress that their values are only a lower bound to T_{m}); our agreement with the Williams *et al.*²⁵ values is close. Our curve falls significantly below the T_{m} measurements of Yoo *et al.*²⁶ (however, the difficulties of obtaining temperature in shock experiments are well known) in which temperature was deduced by measuring optical emission, but accords quite closely with the Brown and McQueen²⁷ value of T_{m} obtained by estimating the shock tempera-

ture using thermodynamic calculation. There is theoretical evidence that the Brown–McQueen temperature may be more reliable¹². Our T_{m} of 6,670 K at the ICB pressure is close to the values inferred from some previous estimates^{2,28}, and gives evidence against the much higher or lower estimates sometimes proposed^{2,25}. The entropy and volume of fusion predicted by our calculations will be reported elsewhere (D.A., G.D.P. and M.J.G., manuscript in preparation).

How reliable is our melting curve? For the given GGA expression for electronic exchange-correlation energy, our exhaustive tests on all sources of technical and statistical error indicate a precision of better than 15 meV per atom on the separate Gibbs free energies of solid and liquid, equating to a precision of 300 K on T_{m} , which is at least as good as current experimental errors. To evaluate the accuracy of GGA itself, we have made detailed comparisons with shock Hugoniot and bulk sound-speed data²⁷ for both solid and liquid, and we find the same close agreement reported in earlier *ab initio* work¹³ on the close-packed solid. Given the accuracy of the *ab initio* low-temperature pressure–volume curve and of phonon frequencies at ambient pressure, we believe that the GGA error in the difference of free energy between solid and liquid is unlikely to be more than 20–30 meV; this implies an error in T_{m} of ~ 300 K. We therefore believe that our predictions for T_{m} are good to ± 600 K. This suggests that they are consistent with the DAC T_{m} values of Williams *et al.*²⁵ and the lower bounds of Shen *et al.*³, but not with the melting curve of Boehler²⁴; at high pressures, we are consistent with the T_{m} of Brown and McQueen²⁷, and just within range of the T_{m} of Yoo *et al.*²⁶.

Light impurities in the Earth's core will shift the temperature at the ICB away from T_{m} for pure Fe. The most likely impurity candidates are sulphur, oxygen, silicon, and perhaps carbon and hydrogen. Apart from our ignorance of the impurity content, the main problem in estimating the ICB temperature is our inadequate knowledge of the thermodynamic properties of solid and liquid alloys of Fe with these impurities at Earth's core conditions. We believe that extension of our *ab initio* techniques will lead to progress here. *Ab initio* thermodynamic integration can be used to calculate the chemical potentials of iron and sulphur in liquid and solid Fe–S alloys as a function of P , T and concentration (D.A. *et al.*, unpublished results). Knowledge of the chemical potentials enables the determination of the separation of a given impurity between solid and liquid phases, and the associated shift of T_{m} .

We have thus presented parameter-free fully *ab initio* calculations of the melting properties of iron in the pressure range 50–350 GPa. The results support some of the experimental measurements of T_{m} over this range, and give a T_{m} value of 6,670 K at the pressure of the inner-core boundary. The *ab initio* techniques for calculating thermodynamic properties under extreme conditions are expected to find application to many other problems concerning the Earth's deep interior. □

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Stable isotope evidence for the food web consequences of species invasions in lakes

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Species invasions pose a serious threat to biodiversity and native ecosystems^{1,2}; however, predicting and quantifying the impacts of invasive species has proven problematic^{3–6}. Here we use stable isotope ratios to document the food-web consequences of the invasion of two non-native predators, smallmouth bass and rock bass, into Canadian lakes. Invaded lakes had lower littoral prey-fish diversity and abundance than uninvaded reference lakes. Consistent with this difference, lake trout from invaded lakes had more negative $\delta^{13}\text{C}$ values (–29.2‰ versus –27.4‰) and reduced trophic positions (3.3 versus 3.9) than those from reference lakes, indicating differences in food-web structure. Furthermore, a comparison of the pre- and post-invasion food webs of

two recently invaded lakes showed that invasion was followed by substantial declines in littoral prey-fish abundance and the trophic position of lake trout, reflecting a shift in the diet of lake trout towards zooplankton and reduced dependence on littoral fish. This study demonstrates the use of stable isotope techniques to detect changes in food-web structure following perturbations; in this instance, bass-induced food-web shifts may have severe consequences for native species and ecosystems.

Human dominance over the Earth's ecosystems has been accompanied by the widespread introduction of exotic species, which has led to the extinction of native species, the collapse of native fisheries and the loss of ecological integrity and ecosystem functioning^{1,7,8}. Ecologists are far from being able to predict, detect or measure the ecological impacts of species invasions^{3–6}. This is not surprising because natural food webs are variable and complex^{9,10}, and using traditional methods to examine the impact of species invasions on aquatic food webs would be laborious, difficult and costly.

Natural stable-isotope distributions are increasingly used to provide a time-integrated measure of food-web relationships based on energy flows. Stable nitrogen isotope ratios ($\delta^{15}\text{N} = (^{15}\text{N}/^{14}\text{N}_{\text{sample}} - ^{15}\text{N}/^{14}\text{N}_{\text{reference}}) / ^{15}\text{N}/^{14}\text{N}_{\text{sample}} \times 1000$) become enriched by 3–4‰ between prey and predator tissues, thereby providing a measure of consumer trophic position^{11–13}. Stable carbon isotope ratios ($\delta^{13}\text{C}$) exhibit little or no trophic level enrichment (<1‰), and are useful for identifying the sources of production for consumers in lakes because benthic algae are typically enriched in $\delta^{13}\text{C}$ relative to phytoplankton^{14,15}.

Here we use stable isotope techniques to quantify the food-web consequences of recent invasions of smallmouth bass (*Micropterus dolomieu*) and rock bass (*Ambloplites rupestris*) in Canadian lakes. Smallmouth bass have been intentionally introduced (both authorized and unauthorized) into aquatic ecosystems well beyond their native range in North America, and on virtually every continent^{16,17}. Furthermore, both bass species may be inadvertently introduced into aquatic ecosystems by the dumping of unused live bait¹⁸, and both are adept at dispersing throughout drainage systems. Consequently, both species have greatly expanded their geographical range over the last century.

These two bass species are presently invading a number of relatively pristine lakes in North America's Northern Hardwood–Boreal Forest transition zone, many of which contain lake trout

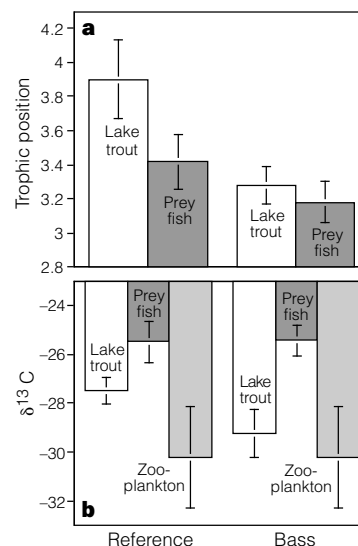


Figure 1 Trophic position and $\delta^{13}\text{C}$ values. **a**, Comparison of mean trophic position of lake trout and pelagic forage fish from invaded and reference lakes. **b**, Comparison of mean $\delta^{13}\text{C}$ values of lake trout, littoral prey-fish and zooplankton from invaded and reference lakes. Error bars represent 1 s.e.m. using lake-specific averages.

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Table 1 Lake and prey-fish data

Lake	Location		Lake area (ha)	Average secchi depth (m)	Total no. of prey species	No. of prey species (in traps)	Prey catch rate (fish per trap per day)	Prey catch rate (grams per trap per day)
	North latitude	West longitude						
Unimpacted								
Louisa	45° 28'	79° 29'	489	7.7	11	7	5.5	36.6
Havelock	45° 17'	78° 37'	175	9.8	5	4	2.6	14.5
Source	45° 33'	78° 39'	271	7.6	8	7	9.8	56.3
MacDonald (1980s)	45° 14'	78° 34'	138	9.8	9	n.d.	n.d.	n.d.
Clean	45° 15'	78° 02'	160	11.7	8	3	0.6	6.5
Mean			268	8.7	8.2	5.3	5.9	35.8
Impacted								
MacDonald (1990s)	45° 14'	78° 34'	138	9.8	2	0	0.0	0.0
Dickie	44° 47'	77° 45'	208	5.9	4	1	1.7	8.9
Johnson	45° 16'	78° 37'	151	7.6	2	0	0.0	0.0
Kelly	45° 15'	78° 37'	99	7.2	2	0	0.0	0.0
Hapy Isle	45° 45'	78° 30'	536	9.9	2	2	3.0	24.4
Mean			226	8.1	2.4**	0.6**	0.9*	6.6*

Background data for the study lakes: lake area (ha), mean summer secchi disk transparency (m), total number of prey-fish species recorded, number of prey-fish species collected in quantitative minnow trapping, prey-fish catch rate, expressed as fish per trap per day and grams per trap per day. Clean lake is undergoing bass invasion at present and is treated as an unimpacted lake as it has not yet undergone a complete invasion.

* $P < 0.05$ between invaded and reference lakes (one-tailed t -test, 7 d.f.).

** $P < 0.001$ between invaded and reference lakes (one-tailed t -test, 7 d.f.).

(*Salvelinus namaycush*) as the native top predator. Although lake trout are generally considered to be a cold-water, pelagic piscivore¹⁹, populations from lakes lacking pelagic prey-fish can consume substantial amounts of fish from littoral habitats^{20,21}. Considering the potential top-down impacts of warm-water predators such as bass on littoral prey-fish communities^{22,23}, we set out to examine the impact of bass invasion on food-web structure; in particular, the impact on the pathways of energy flow leading to the native top predator, lake trout.

Lakes invaded by bass contained fewer prey-fish species than reference lakes in which bass have not become established (Table 1; one-tailed t -test, $t = 5.53$, $P < 0.0005$). Similarly, our minnow-trapping efforts in invaded lakes produced fewer prey-fish species ($t = 4.59$, $P < 0.0025$) and lower prey-fish catch rates (fish per trap

per day, $t = 1.95$, $P < 0.05$; grams per trap per day, $t = 1.94$, $P < 0.05$) than in reference lakes.

We investigated whether these differences in the prey-fish communities between invaded and reference lakes are consistent with food-web differences among lakes, as inferred from natural stable-isotope distributions in lake trout tissues. Habitat- and lake-specific differences in $\delta^{15}\text{N}$ values at the base of the food web preclude the use of consumer $\delta^{15}\text{N}$ values as an absolute measure of consumer trophic position. Instead, the trophic position of consumers can be estimated by interpreting consumer tissue $\delta^{15}\text{N}$ values relative to the $\delta^{15}\text{N}$ of primary consumers, which are used as indicators of 'baseline' isotopic values^{13,24}.

The trophic position of lake trout averaged 3.3 in invaded lakes, indicating a plankton-based diet, compared with 3.9 in reference lakes, indicative of a primarily fish-based diet (t -test, $n = 10$, $t = 2.40$, d.f. = 8, $P < 0.025$; Fig. 1a). A two-source mixing model²⁵ using lake-specific mean trophic-position data and the zooplankton trophic-position endpoint of 2.0 (estimate based on 30 zooplankton samples) indicated that the diet of lake trout from reference lakes was, on average, 62% fish, compared to 22% in invaded lakes (Table 2).

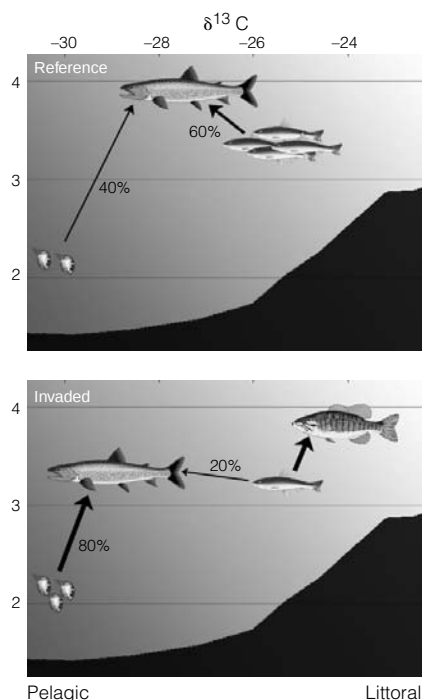


Figure 2 The pathways of energy flow through food webs of reference lakes (lakes in which bass have not become established) and lakes invaded by smallmouth bass and rock bass, based on $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ information.

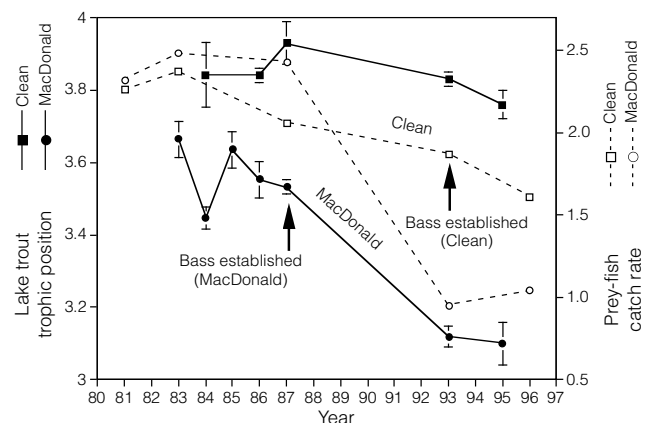


Figure 3 Temporal changes in the trophic position of lake trout (left axis) and log-transformed littoral prey-fish catch rate from quantitative electrofishing transects (right axis) in MacDonald Lake (circles) and Clean Lake (squares) for the period 1981–1996. Arrows indicate the year in which both smallmouth bass and rock bass were fully established in MacDonald and Clean lakes.

Table 2 Trophic position, $\delta^{13}\text{C}$ and mixing-model results

Lake	Lake trout trophic position (± 1 s.d.)	Prey fish trophic position (± 1 s.d.)	Lake trout diet		Lake trout δ ¹³ C (‰) (± 1 s.d.)	Prey fish δ ¹³ C (‰) (± 1 s.d.)	Pelagic δ ¹³ C (‰)	Lake trout diet	
			% Fish	% Zooplankton				% littoral	% pelagic
Unimpacted									
Louisa	4.82 (0.25)	3.84 (0.52)	99	1	-25.82 (0.35)	-25.31 (2.15)	-29.70	88	12
Havelock	3.73 (0.07)	3.01 (0.30)	70	30	-27.77 (0.60)	-27.63 (1.41)	-29.99	93	7
Source	3.60 (0.26)	3.21 (0.22)	46	54	-28.26 (1.12)	-26.54 (0.87)	-29.99	50	50
MacDonald (1980s)	3.55 (0.14)	3.26 (0.22)	40	60	-28.79 (1.25)	-25.60 (4.24)	-30.92	40	60
Clean	3.82 (0.18)	3.76 (0.26)	44	56	-26.75 (0.98)	-22.45 (1.33)	-29.58	40	60
Mean	3.90	3.42	64	36	-27.48	-25.50	-30.04	62	38
Impacted									
MacDonald (1990s)	3.11 (0.21)	3.26 (0.22)	3	97	-29.55 (1.02)	-25.60 (4.24)	-30.92	26	74
Dickie	3.18 (0.39)	2.92 (0.07)	12	88	-32.59 (1.43)	-27.49 (0.90)	-33.59	16	84
Johnson	3.71 (0.29)	3.55 (-)	43	57	-26.62 (1.26)	-23.65 (-)	-28.36	37	63
Kelly	3.29 (0.34)	3.23 (0.30)	18	82	-28.83 (0.93)	-25.40 (2.32)	-28.36	31	69
Happy Isle	3.12 (0.33)	2.93 (0.01)	5	95	-28.39 (1.49)	-25.02 (0.41)	-30.39	26	74
Mean	3.28	3.18	16	84	-29.20	-25.43	-30.32	27	73

Stable isotope-based estimates of trophic position of lake trout and littoral prey fish are shown. Two-source mixing models use the average zooplankton trophic position of 2.0 and lake-specific mean trophic position values to estimate the % fish and % zooplankton contributions to lake trout diet. Mean lake trout, littoral prey fish and pelagic (zooplankton and unionid mussel) $\delta^{13}\text{C}$ are also presented. Mixing models were used to estimate the % contribution of littoral and pelagic prey to lake trout diet. The $\delta^{13}\text{C}$ mixing models for Havelock and Source lakes use the cross-lake mean pelagic $\delta^{13}\text{C}$ value as the pelagic endpoint owing to the lack of lake-specific data.

$\delta^{13}\text{C}$ signatures of lake trout muscle tissues provide additional evidence for food-web differences between invaded and reference lakes. $\delta^{13}\text{C}$ in lake trout from reference lakes averaged -27.5‰ , indicative of reliance on littoral prey, while $\delta^{13}\text{C}$ in lake trout from invaded lakes was -29.2‰ , indicating greater use of pelagic prey (t -test, $n = 10$, $t = 1.55$, d.f. = 8, $P < 0.10$; Fig. 1b). Two-source mixing models using lake-specific pelagic (mean of zooplankton and unionid mussel) and littoral prey-fish $\delta^{13}\text{C}$ endpoints indicated that lake trout from reference lakes averaged 62% littoral prey, compared with only 27% for lake trout from invaded lakes (Table 2). Because differences in '% fish' and '% littoral' were so closely coupled (% littoral = $9.69 + 0.85 \times \text{% fish}$, $n = 10$, $P < 0.001$, $r^2 = 0.84$), both lines of evidence were used simultaneously to summarize the differences in food-web structure between invaded and reference lakes (Fig. 2).

Our comparison of invaded and reference lakes is complemented by long-term studies of MacDonald and Clean Lakes. These two lakes are close to each other (<200 m), and were nearly identical in lake area, morphometry, littoral habitat and fish species composition before bass invasion. Both lakes have now been invaded by the two bass species, although the chronology of bass establishment differs for the two lakes. In MacDonald lake, both bass species were fully established by 1987, after which littoral prey-fish catch rates declined markedly (Fig. 3). Corresponding with this decline in the littoral prey-fish community, lake trout from MacDonald Lake exhibited a marked decline in trophic position following bass establishment. In Clean Lake, smallmouth bass were found in small numbers during the 1980s, although it was not until 1993 that both bass species had become fully established. The data from Clean Lake indicate that a reduction in the littoral prey-fish community and the trophic position of lake trout has begun (Fig. 3).

Pelagic and littoral habitats are often treated as separate components of lake ecosystems²⁶. In this study, a native top predator generally considered to be pelagic¹⁹ was sustained primarily by littoral resources, thereby linking these two habitats. Furthermore, the magnitude of this littoral–pelagic habitat coupling was mediated by strong top-down effects of invasive predators, resulting in an unexpected interaction between cold-water and warm-water fish species. Our findings contribute to a growing body of evidence that subsidies of resources from other systems or habitats can be both energetically significant and important in food-web dynamics¹⁰.

The food-web changes described here have severe implications for native lake trout populations (D.M. Brown and J.M.C.,

unpublished data). However, not all lake trout populations are expected to be affected similarly by bass invasions, and the extent to which an invasion affects lake trout should depend on the structure of the pre-invasion food web. Specifically, bass–trout interactions should be minimal in lakes containing pelagic prey fish (such as cisco, smelt and alewife) because pelagic prey-fish species are generally not consumed by bass, but are a major prey item of lake trout²⁰. It is lakes lacking pelagic forage fish, such as those examined here, that are particularly affected by invasive predators.

Increased understanding of lake trout–bass interactions might have implications for fishery management in the many other regions in which non-native predators are deliberately introduced into aquatic ecosystems. Contrary to the prevalent belief that introduction of bass and other predators represents a form of fishery enhancement, our data draw attention to the adverse competitive impacts of introduced predators upon native fish populations. Protecting native fish populations will often necessitate halting the intentional introduction of bass and other predators, and limiting the use of live bait by anglers.

Predicting the impact of perturbations such as species invasions in natural food webs presents a formidable challenge to ecology. It is increasingly recognized that broad-scale, ecosystem-level approaches (both experimental and comparative) are crucial, and even uniquely required to understand and predict ecosystem-level processes^{27,28}. This study demonstrates how the use of stable isotopes can provide time-integrated and energy-based depictions of food-web structure. In fact, this isotopic approach provides a very sensitive indicator of environmental change that can be used to quantify the impacts of a broad range of anthropogenic activities on food-web structure and the pathways of energy flow in natural ecosystems. □

Methods

All field sampling was conducted from 1995 to 1997. Lake trout and littoral prey-fish were collected using gill nets, minnow traps, beach seines and from anglers. Zooplankton were collected using a 250- μm zooplankton net. Details of the isotopic analysis are presented elsewhere¹³. The trophic position of each fish was estimated using muscle tissue $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ data and a baseline correction method that interprets the fish $\delta^{15}\text{N}$ value relative to that of the lake- and habitat-specific primary consumer $\delta^{15}\text{N}$; this value is used as the 'baseline' for estimating trophic position¹³. Consumer trophic position is estimated using the formula: Trophic position_{consumer} = $((\delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{baseline}})/3.4) + 2$, where 3.4 is the assumed per mil level increase in $\delta^{15}\text{N}$; primary producers = trophic level 1, primary consumers = trophic level 2, and so on. Trophic position estimates for lake trout 1983–93 rely on the 1995 baseline data. Our analysis is based on the measurement of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ signatures from 433 samples from the nine study lakes.

Species were considered to be littoral prey-fish if they have been found to be, or are likely to be, consumed by lake trout²⁰. Littoral prey-fish catch rates (Table 1) are expressed as number per trap per day and grams per trap per day, and were estimated from 24-h sets of baited minnow traps (15–40 traps per lake) during 1996 and 1997. The littoral prey-fish catch rate shown in Fig. 3 is the log-transformed catch rate (biomass) from 100-m quantitative electrofishing transects.

Counts of littoral prey-fish species are based on our field sampling efforts, unpublished Ontario Ministry of Natural Resources documents and quantitative electrofishing excursions on MacDonald and Clean lakes. Owing to the higher efficiency of electrofishing for detecting species, only the littoral prey-fish species that make up more than 1% of the electrofishing catch were retained in the species lists of these two lakes²⁹.

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Stable isotopes reveal strong marine and El Niño effects on island food webs

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Stable isotope analysis is a powerful tool for unravelling the complex structure of food webs^{1–3}. This technique is particularly well suited for studies at ecosystem boundaries, where physical processes and mobile consumers link the dynamics of seemingly disparate systems^{4–6}. In coastal and insular environments, seabirds play a crucial role in transporting marine-based energy and nutrients to islands^{7–9}. Here we show using stable isotopes that nutrients from the ocean drive the dynamics of terrestrial food webs on small islands. The indirect effects of seabird-derived nutrients on plant productivity are particularly prominent during wet El Niño Southern Oscillation years on our Gulf of California study sites. During dry years that characterize the region, many terrestrial consumers are subsidized by carrion and prey from the ocean. Shifts in trophic structure related to El Niño Southern Oscillation could only be elucidated because of the distinct nitrogen isotope ratios associated with seabird islands. The contributions of seabirds and other marine sources are reflected in the isotope signatures of terrestrial consumers in ways that challenge conventional interpretations of stable isotope results in studies of food webs.

For the last 10 years, we have studied the effects of allochthonous resources from the ocean on terrestrial communities of islands off Baja California, Mexico (28° 55' N, 113° 30' W)^{10–14}. These islands are situated in one of the driest and most barren regions of North America (mean annual precipitation, 59 mm), and marine inputs are an important source of energy and nutrients. These inputs take two forms. First, tens of kilograms of detrital algae and carrion wash ashore per metre of shoreline each year, supporting a rich intertidal community and abundant terrestrial scavengers, detritivores and predators at and above the supralittoral zone¹⁰. Second, seabirds that roost or nest on smaller islands provide food for terrestrial scavengers in the form of food scraps and carcasses. Seabirds also deposit large amounts of guano, which fertilizes the soil and stimulates primary productivity^{7,14–16}. Growth of plants, especially annuals, is particularly striking on seabird islands during periodic El Niño Southern Oscillation (ENSO) events that bring heavy precipitation. This flush of vegetation seems to switch the system from one that is dependent primarily on allochthonous input from the ocean to one that is driven more by *in situ* terrestrial productivity. Terrestrial arthropods track these changes: in dry years, aerial insect assemblages are dominated by taxa associated with the ocean (kelp, carrion and parasitic flies)¹⁰, but herbivores, which depend on terrestrial plants, increase 40–190-fold during ENSO years¹¹.

Stable isotope analysis is increasingly used to examine the exchange of energy and nutrients between ecosystems, especially at the ocean–land interface^{1,17,18}. Stable isotope ratios of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$), expressed as the ratio of heavy-to-light carbon ($^{13}\text{C}/^{12}\text{C}$) and nitrogen ($^{15}\text{N}/^{14}\text{N}$) relative to Pee Dee belemnite limestone and atmospheric N standards, respectively, are the most widely used. $\delta^{13}\text{C}$ values of consumers in marine phytoplankton food webs are significantly enriched (that is, higher in ^{13}C) compared with those in food webs based on plants with C3 photosynthesis, but usually less enriched than consumers in C4 and CAM (crassulacean acid metabolism) plant-dominated environments¹. Because relatively little ($\leq 1\%$) fractionation of C isotopes occurs between a consumer and its prey, a consumer's $\delta^{13}\text{C}$ signature is similar to that of its diet. In contrast, $\delta^{15}\text{N}$ is thought to

increase by 3–5‰ with each increase in trophic level, potentially making $\delta^{15}\text{N}$ a useful indicator of trophic position^{19,20}. Because consumer $\delta^{15}\text{N}$ ultimately builds on the nitrogen signature of food sources at the base of the web, $\delta^{15}\text{N}$ can complement $\delta^{13}\text{C}$ in distinguishing between multiple possible diet sources^{19,21}.

We compared the C and N isotope signatures of representative organisms from seabird-roosting islands (SB islands) with those of the same species on neighbouring islands where no or few seabirds were present (NB islands). Our 1998 field work coincided with the strong 1997–1998 ENSO event, which brought roughly 440 mm of precipitation to nearby Bahía de los Angeles from September 1997 to August 1998, and ended a two-year drought during which only 2 mm of rain fell. In addition to terrestrial plants and animals, we also analysed samples from algae and intertidal invertebrates and insects eating fish carrion to obtain isotope signatures of potential marine-based foods.

To investigate the effects of ENSO-related precipitation, we analysed samples collected in May and June 1997 of two abundant groups that consumed both terrestrial and marine resources. Tenebrionid beetles comprise about 70% of ground-dwelling macroarthropods (excluding ants) on Gulf islands and eat both plant detritus and carrion⁹. *Peromyscus maniculatus*, an omnivorous rodent, eats mostly seeds and terrestrial arthropods, but also forages extensively for intertidal invertebrates^{22,23} (P.S. and G.A.P., manuscript in preparation).

Figure 1 shows the C and N isotope signatures of plants and consumers on SB and NB islands, as well as those of intertidal and carrion-feeding organisms. On both types of island, C3 and C4/CAM plants represent the ends of a continuum of terrestrial C sources; marine carrion is intermediate in $\delta^{13}\text{C}$ and intertidal organisms overlap with C4/CAM plants. However, the most striking pattern in Fig. 1 is the marked difference in $\delta^{15}\text{N}$ signatures of plants

and consumers on SB compared with NB islands. Mean values of consumer $\delta^{15}\text{N}$ from our SB islands are the highest yet reported for any terrestrial or marine animals, exceeding even those of top-level mammalian carnivores^{2,19}. Previous studies showed the significant $\delta^{15}\text{N}$ enrichment of soils in seabird colonies (>40‰; ref. 24; our SB islands, soil $\delta^{15}\text{N} = 28.26 \pm 5.44$, $n = 4$) due to isotope fractionation during microbial conversion of uric acid in guano ($\delta^{15}\text{N} = 14.23 \pm 2.34$, $n = 6$) to ammonia, which is subsequently volatilized^{16,24}. As a consequence, plants and algae fertilized by seabird guano tend to have relatively high $\delta^{15}\text{N}$ signatures^{8,14,24}. Therefore, although marine resources are responsible for much of the N entering the food web on SB islands in wet years, the effects of seabirds are channelled through soil and plants to higher-level consumers, and are largely indirect.

Likewise, stable isotopes revealed that food webs of NB islands in wet 1998 were primarily based on terrestrial plants. Herbivores and detritivores (Acrididae grasshoppers (gh), Tenebrionidae beetles (te) and silverfish (sl)) consumed a mixture of C3 and C4/CAM plants, whereas *Chaetodipus baileyi* (cb), a granivorous rodent present only on NB islands, apparently ate relatively more C4/CAM seeds (Fig. 1). One explanation for the enriched $\delta^{13}\text{C}$ signature of *Chaetodipus* is that reproduction of the dominant plant, *Atriplex barclayana*, a C4 shrub that comprises 44% and 84% of perennial plant cover on NB and SB islands, respectively, was three times greater in 1998 than in the previous dry year (A. Subalusky and G.A.P., unpublished data). $\delta^{15}\text{N}$ of *Peromyscus* (pm) was much higher than that of *Chaetodipus* but similar to that of ground spiders (sp; Fig. 1), indicating that *Peromyscus* may not be a strict granivore on these islands but feeds mostly on other consumers. Although the 2.9–5.4‰ difference in $\delta^{15}\text{N}$ between spiders and their presumed prey (silverfish (sl)) is consistent with the widely documented trophic effect, there was no evidence of stepwise enrichment in $\delta^{15}\text{N}$ between plants and herbivores. Similar results have been reported^{13,25,26}, but the N isotope fractionation associated with herbivory clearly requires additional study.

ENSO events such as that in 1998 occur infrequently. Our correlational work^{10,11} indicated that, during dry years typical of the region, many terrestrial consumers rely heavily on marine-derived prey. Our analyses of tenebrionid beetle and *Peromyscus* from dry 1997 support this conclusion, but these groups responded differently on SB and NB islands (Fig. 2). On NB islands, the diet of *Peromyscus* in 1997 was similar to that in wet 1998 ($\delta^{13}\text{C}$: t -test = -1.42, degrees of freedom (d.f.) = 33, $P = 0.17$; $\delta^{15}\text{N}$:

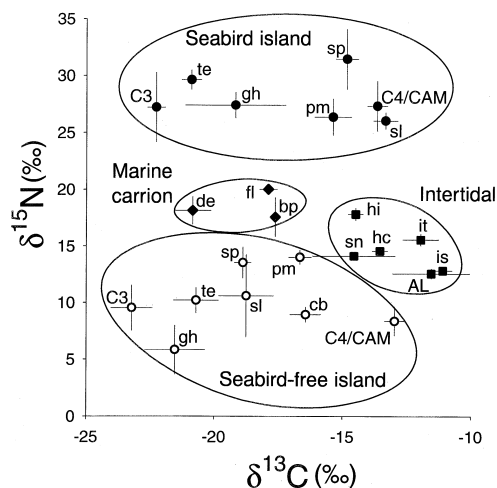


Figure 1 Carbon and nitrogen stable isotope signatures (mean $\pm$ 1 s.e.) of food webs associated with SB islands (filled circles), NB islands (open circles), the intertidal zone (filled squares) and marine carrion (filled diamonds) during 1998, a wet El Niño year. Abbreviations (with sample sizes for 1998 for SB, NB islands, respectively): C3 plants (*Coreocarpus*, *Camissonia*, *Encelia*, *Nicotiana*, *Chenopodium*, *Perityle*, *Viscainoa*, *Mojavea*; 5, 9); C4/CAM plants (*Amaranthus*, *Atriplex*, *Mesembryanthemum*, *Ferocactus*, *Pachycereus*, *Opuntia*; 10, 5); terrestrial herbivores: gh, Acrididae grasshoppers (4, 4), cb, granivorous rodent (*Chaetodipus*; 0, 17); terrestrial detritivores: te, Tenebrionidae (*Argoporis*, *Triphalopsis*) beetles (6, 6), sl, Thysanura silverfish (3, 3); terrestrial predators: sp, Agelenidae ground spiders (2, 3), pm, omnivorous rodent (*Peromyscus*; 8, 22). Scavengers/carrion feeders: de, Dermestidae (*Dermestes*) beetles (8), fl, Sarcophagidae fly larvae (8). Intertidal community: AL, macroalgae (3 spp.; 7); intertidal detritivores: it, *Phaleria* (Tenebrionidae) beetle (3), is, *Ligia* isopod (8), hc, *Paguristes* hermit crab (4), sn, *Thais* snail (4); intertidal predator: hi, *Baekmaniculus* (Histeridae) beetle (3). Seabird (that is, marine-based carrion): bp, brown pelican¹³. Sample sizes for 1997 (SB, NB islands): Tenebrionidae beetles (4, 6), *Peromyscus* (8, 13).

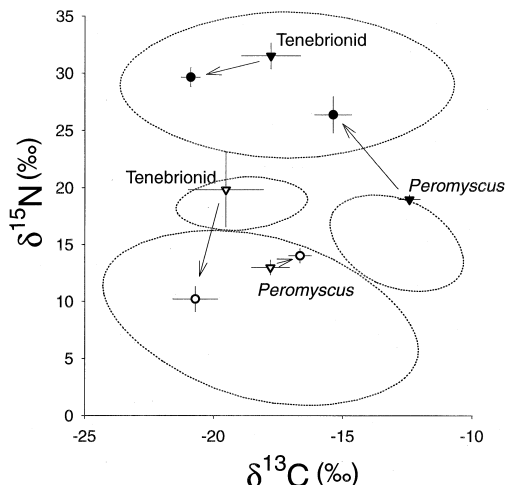


Figure 2 Shift in carbon and nitrogen isotope signatures (mean $\pm$ 1 s.e.) of omnivorous rodents (*Peromyscus*) and tenebrionid beetles from a typical dry year (1997; triangles) to a wet El Niño year (1998; circles). Filled symbols are SB islands; open symbols are NB islands. Ellipses indicate the four different food webs in Fig. 1.

$t = -1.08$, d.f. = 33, $P = 0.29$, but beetles fed at a higher trophic position (that is, higher $\delta^{15}\text{N}$) in 1997 ($t = 2.78$, d.f. = 10, $P = 0.02$). Comparing the 1997 isotopic signature of beetles with the 1998 values in Fig. 1, the most likely explanation for the higher $\delta^{15}\text{N}$ in 1997 is an increase in consumption of animal tissue, probably marine carrion. In contrast, on SB islands, tenebrionids apparently did not alter trophic position, scavenging plant detritus and terrestrial carrion in both years ($\delta^{15}\text{N}$: $t = 1.34$, d.f. = 8, $P = 0.22$), although the significant shift in $\delta^{13}\text{C}$ ($t = 3.07$, d.f. = 8, $P = 0.02$) indicated that they relied more on C4/CAM-derived material in 1997 (Fig. 2).

The difference in isotopic signature of *Peromyscus* between 1997 and 1998 underscored the overwhelming importance of ocean resources on small SB islands. In wet 1998, $\delta^{15}\text{N}$ of *Peromyscus* was equivalent to that of guano-fertilized terrestrial plants, suggesting a diet of mostly seeds (Fig. 1). During the previous dry year, however, mice from the same inland locations fed extensively on intertidal organisms, as reflected in the significant shift in both $\delta^{13}\text{C}$ ($t = 3.60$, d.f. = 14, $P = 0.003$) and $\delta^{15}\text{N}$ ($t = -4.50$, d.f. = 14, $P < 0.001$; Fig. 2). This conclusion is supported by *Peromyscus* foraging behaviour: mice moved further and more frequently between shore and inland on SB islands than on NB islands, and were more abundant near shore than inland (P.S. and G.A.P., manuscript in preparation). We suspect that, on small roosting islands, marine-derived foods are critical for survival and persistence of *Peromyscus*, as well as other consumers during extended dry periods. For example, web-building spiders apparently consume carrion insects (mean $\delta^{13}\text{C} = -20.3$, $\delta^{15}\text{N} = 22.0$, $n = 5$) during dry years, when aerial herbivores are scarce.

The shift in consumer isotope signatures between 1997 and 1998 also demonstrates several pitfalls (and strengths) of stable-isotope studies, with important implications for interpretation of these studies in other systems. First, the difference in *Peromyscus* $\delta^{15}\text{N}$ between years is, at first glance, counterintuitive, based on traditional interpretations of $\delta^{15}\text{N}$ as an indicator of trophic level. The decrease in trophic position of *Peromyscus* from intertidal predator (1997) to granivore (1998) was actually reflected by an increase, not a decrease, in $\delta^{15}\text{N}$ (Fig. 2). Herbivores having higher $\delta^{15}\text{N}$ than predators in the same system was unexpected, and questions the utility of comparative studies that rely solely on isotope data to examine trophic relationships across broad, geographic scales. Similarly, the marked change between years in the diet of the same species in exactly the same location reveals the potential risks of assigning functional roles to species based on data from a single year.

Second, had we sampled only terrestrial resources and ignored the potential marine-based foods available to mice in the intertidal or to beetles on NB islands, our 1997 isotope results would have been difficult to interpret. Our study thus underscores the critical importance of identifying and sampling the multiple sources of energy and nutrients available to consumers in food webs²⁷. As in our research, these sources often include substantial contributions from adjacent habitats or ecosystems that enter a focal system by physical processes or the activities of mobile organisms⁵. Although ecosystem and aquatic ecologists have long recognized the importance of allochthonous energy and nutrients^{4,5}, we propose that the consequences of these inputs for terrestrial populations and food webs have, to date, been underappreciated. Finally, the distinct isotopic signatures associated with marine sources allowed us to appreciate the interdependency of the land and ocean in structuring insular food webs, and to detect shifts in trophic roles of consumers in response to changes in terrestrial productivity. Increasingly, researchers have taken advantage of unique source signatures associated with agricultural or urban pollutants to examine the effects of these inputs on local nutrient and community dynamics^{28–30}. Failure to recognize the importance of these inputs and their effects on interpretation of stable isotope results could lead

to erroneous conclusions about trophic relationships and dynamics in food web studies. □

Methods

We analysed leaf and seed tissue from plants and tissues from whole (small arthropods) or partial (exoskeleton of large invertebrates; rodent tails) animals on three SB islands (Coronadito, Flecha, Llave) and four NB islands (Ventana, Cabeza de Caballo, Pata, Smith). All terrestrial specimens were collected >50 m from shore. Samples from 1997 were collected from inland locations on the same islands as in 1998. Intertidal organisms were collected along island and peninsula shores, whereas carrion-feeding arthropods were collected from traps baited with fish.

Animal samples were stored in 70% ethanol; rodent tissues were also frozen upon return from the field. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of ground, dried samples were determined using Europa mass spectrometers connected to Europa C–N analysers at Berkeley and at Davis, University of California.

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Built-in polarizers form part of a compass organ in spiders

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Some insects and vertebrates use the pattern of polarized light in the sky as an optical compass^{1–5}. Only a small section of clear sky needs to be visible for bees and ants to obtain a compass bearing for accurate navigation^{5,6}. The receptors involved in the polarization compass are confined to a small part of the retina, and the eyes are built predominantly for other visual tasks⁷. Here we report the discovery of a unique compass organ in the spider *Drassodes cupreus*, where a pair of specialized secondary eyes cooperate to analyse skylight polarization. These eyes do not form images, but use a built-in polarization filter to determine precisely the direction of polarization. Measurements using a model eye indicate that the compass organ is best suited for navigation at dusk and dawn. Behavioural experiments show that the spiders are primarily active after sunset and that they use polarization cues to find their way back to the nest after foraging trips. A similar organization of the secondary eyes in several spider families indicates that such compass organs may not be an isolated phenomenon.

Arthropod photoreceptors are intrinsically sensitive to the direction (E-vector) of polarized light. This is because the photopigment lies in the membranes of densely packed microvilli that predominantly absorb light with an E-vector parallel to their long axis^{8,9}. If all the microvilli of a photoreceptor are straight and aligned, the cell as a whole exhibits a strong preference for a particular E-vector. By comparing the response from cells with different microvillar orientations, arthropods can compute the direction of the E-vector^{10,11}. In the specialized dorsal-rim area of many insect compound eyes, this ability is used for analysing the polarization pattern of the blue sky, which changes according to the position of the Sun and thus provides compass bearings for navigation¹². A few spiders^{13,14} use their principal eyes for the same purpose. The secondary eyes of spiders, of which there are normally three pairs, have not been implicated in any visual tasks involving polarization cues. However, we have found that a pair of secondary eyes (the postero-medial (PM) eyes) of a gnaphosid spider, *Drassodes cupreus* (Blackwall), polarize the light returning from its reflecting tapetum. To our knowledge, polarizing optics have not been demonstrated in the eyes of any animal, and its presence in this spider eye led us to suspect that the PM eyes are specialized for polarization vision.

To investigate this possibility we first examined the structure and optical properties of the eye. The two PM eyes point dorsally and are close together on the dorsal surface of the cephalothorax (Fig. 1a). They have an oval shape, with the long axes oriented roughly at right angles to one another (Fig. 1b). A reflecting tapetum makes the eyes appear brilliantly blue. When illuminated with polarized light, the bright blue glow is nearly extinguished if the plane of polarization is oriented at right angles to the long axis of the eye (Fig. 1c, d). When the plane of incident polarization is rotated by 90°, the extinction moves from one eye to the other. Light polarized along the long axis of the eye is reflected four times more efficiently than light polarized

along the short axis. Pieces of the tapetal mirror isolated in physiological saline act as polarizing reflectors, but no other components of the eye have polarizing properties strong enough to show up in transmitted or reflected light polarization microscopy. Our findings strongly support the idea that the PM eyes are polarization detectors, and that they work as a single organ by comparing the signals from the left and right eyes.

The tapetum is organized as two flat mirrors forming a V-shaped trench with an angle of 90–100° between the sides (Fig. 2a, d). At the bottom of the eye, the two sides are separated by a narrow gap through which the photoreceptor axons emerge. A tapetum with two separated halves is common in spider secondary eyes, and is known as the canoe type of tapetum¹⁵. The function of secondary eyes with canoe-tapeta has been enigmatic because the corneal optics generally lack sufficient dioptric power to produce a focused image on the retina¹⁶. In *D. cupreus* this is taken to an extreme: the eye is effectively lensless and the cornea acts as a mere window exposing the retina to light from the sky. Consequently, the retinal layout is directly visible from outside the intact eye (Fig. 1). The flat

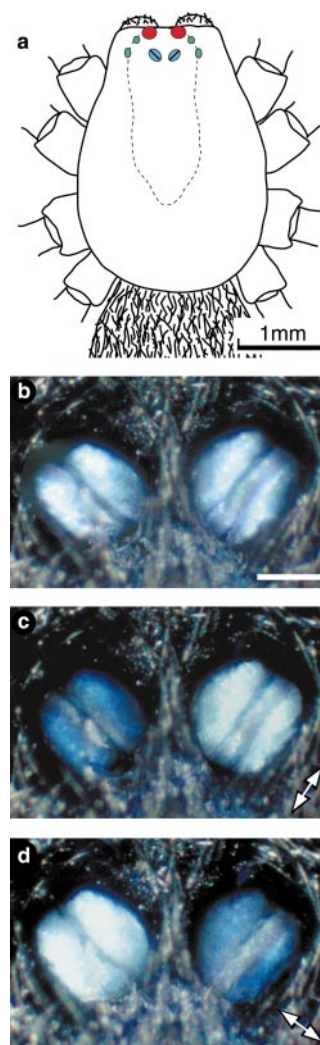


Figure 1 The compass organ of *D. cupreus*. **a**, Drawing of the cephalothorax indicating the position of the principal eyes (red) and three pairs of secondary eyes (blue, PM eyes; green, other secondary eyes). **b**, Close-up view of the pair of PM eyes, with the retinal division directly visible through the lensless corneal window. **c**, **d**, The PM eyes viewed under plane-polarized illumination with the E-vector (arrows) parallel to the long axis of the right (**c**) and left (**d**) eye. The extinction of the opposite eyes shows that there is a built-in polarizer blocking light polarized perpendicular to the long axis of the eye. Scale bar, 100 μm (**b**).

mirrors and the lack of a focusing lens imply that there is no imaging, and that all the retinal photoreceptors have the visual field of the whole eye. Using the blue tapetal reflection as an indicator, the visual fields of the two PM eyes were found to overlap almost entirely, covering a solid cone of roughly 125° centred on the vertical (Fig. 3a, b).

Receptive segments (rhabdomeres) of the visual cells fill the bottom of the eye. We distinguished two types of receptor by their position in the retina and by their microvillar orientation (Fig. 2b, d). About 60 main receptors make up the bulk of the retina and form a characteristic laminar arrangement of rhabdoms, with the microvilli aligned along the long axis of the eye (Fig. 2c). Light entering the eye reaches the rhabdoms along three different paths: from above; after one tapetal reflection; and after two tapetal reflections (Fig. 2d). As the tapetum polarizes light along the long axis of the eye, it will enhance the intrinsic polarization sensitivity of the main receptors, making them ideally suited for polarization detection. Two cells in each eye form shallow receptors that are confined to the distal part of the retina along a central line (Fig. 2b, d). The shallow receptors have less ordered microvilli that are aligned predominantly at right angles to those of the main receptors. The intrinsic polarization sensitivity of the shallow receptors will be reduced by the polarizing tapetum. Consequently, the

shallow receptors are unsuitable for polarization detection and are likely to provide only a luminance signal. Reliable polarization analysis requires a comparison of signals from cells with high polarization sensitivities but different polarization axes¹⁷, and in the present case this can only be obtained by opponent input from the main receptors in the left and right PM eyes.

To confirm the polarization sensitivity (PS) of the main receptors, we made intracellular recordings of their responses to polarized light flashes. Although this proved extremely difficult, we held two cells long enough to measure their PS ratios⁹ *in situ*, providing values of 7.6 and 9.1 (see Supplementary Information), which are high compared with those of dorsal-rim receptors in the eyes of navigating insects (PS = 6.3 in *Cataglyphis* ants¹⁸ and 6.6 in honeybees¹⁹). We held one of the cells (PS = 9.1) long enough to also measure its spectral sensitivity, which peaked in the ultraviolet (350 nm). From the high PS, we assume that the two cells were of the main receptor type. Electrorretinograms revealed two spectral types of cell in the eye, maximally sensitive to green (500 nm) and ultraviolet (350 nm), but there is as yet no confirmation that the green cells are the shallow receptors.

Although the PM eyes of *D. cupreus* have all the properties of a skylight polarization compass, they differ in several respects from the dorsal-trim area of insect compound eyes. One peculiarity is

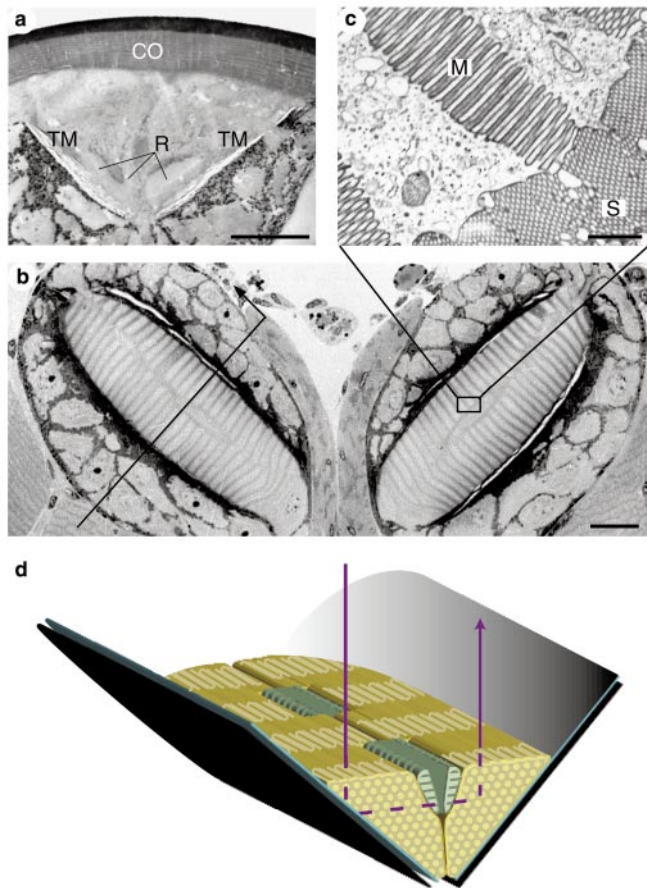


Figure 2 Structure of the PM eyes. **a**, Section (low magnification electron micrograph) through the long axis of the eye showing the almost flat cornea (CO) and the two flat tapetal mirrors (TM). The rhabdoms (R) are only faintly visible. **b**, Tangential section (light micrograph) through the retina of both PM eyes revealing the arrangement of rhabdomer plates of the main receptors and the islands of shallow receptors along the retinal midline. Black line represents site of section in **a**. **c**, Electron micrograph showing the microvillar orientation of the main (M) and shallow (S) receptors. **d**, Drawing of the canoe-shaped tapetum and the retina, with the front end cut off to expose interior structures. The main receptors are coloured yellow, and the shallow receptors green. Scale bars, 20 μm (**a**, **b**); 1 μm (**c**).

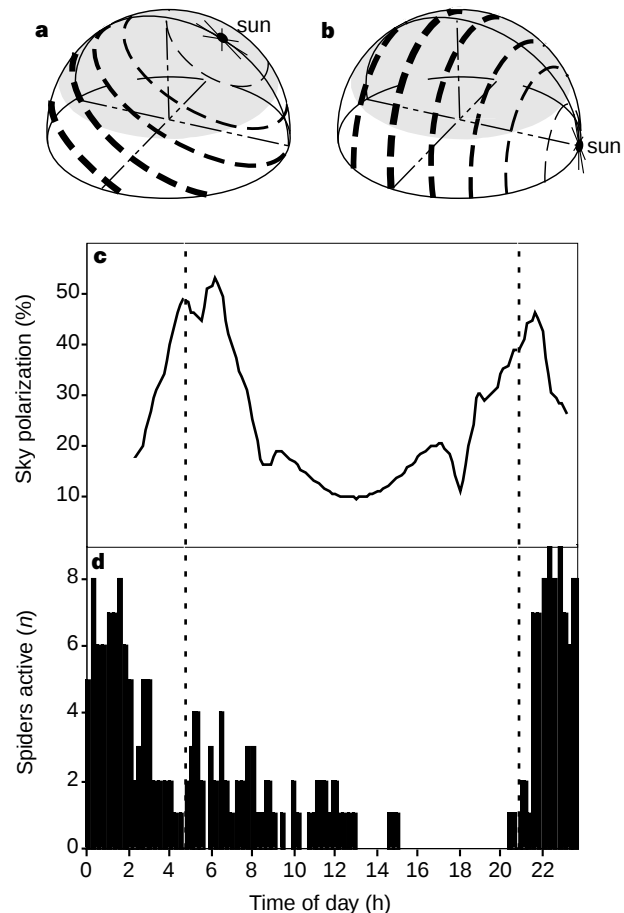


Figure 3 Sky-polarization analysis at dusk and dawn. **a**, **b**, Polarization pattern of the sky hemisphere, with E-vector direction and strength indicated for solar positions high above the horizon (**a**) and at the horizon (**b**). Shaded areas mark the visual field of the PM eyes, which receive a mixture of E-vectors, including direct sunlight, in **a**, but primarily one E-vector in **b**. **c**, Measurements of sky polarization made with a model eye integrating across the same angle as the PM eyes of *D. cupreus*. The measurements were made during 24 h of almost clear weather (sunset and sunrise indicated by dashed lines). **d**, The activity of nine individuals monitored every 15 min for 24 h. High activity correlates with strong sky polarization as perceived by the model eye.

that the PM eyes lack imaging optics and thus integrate over a substantial visual field, that is, already at peripheral level. Sky light is polarized tangentially to the Sun²⁰ (Fig. 3a, b), and when the Sun is high above the horizon a wide-field integrating analyser would receive a mixture of different planes of polarization (Fig. 3a). This, and the input of direct unpolarized sunlight, would tend to obscure the polarization signal. Immediately after sunset, however, the sky is polarized mainly in one direction (Fig. 3b), and no direct sunlight interferes with a wide-field analyser. Thus it seems that the PM eyes are designed for polarization navigation at dusk and dawn. To test this hypothesis, we built a model eye that, like the real PM eyes, measures skylight polarization over a large angle and at short wavelengths. This device was used to monitor the strength of the polarization signal over a number of 24-h periods of mostly clear weather. The outcome (Fig. 3c) confirms that the design of the spider eye is best suited for use at sunset and sunrise. Recordings of the activity pattern of the spiders demonstrate peak activity immediately after sunset (Fig. 3d), thus indicating further that the PM eyes are polarization analysers designed for navigation at dusk.

D. cupreus lives in open habitats and resides in a silk nest spun under a small rock. From this refuge it makes foraging trips and presumably returns to the same nest. To find out whether sky polarization and PM eyes are involved in this behaviour, we placed individual spiders in a circular arena with four symmetrically placed shelters, polarized illumination from above and a faint intensity gradient across the ceiling (Fig. 4). Of ten spiders that made a nest under a shelter within a 24-h period, nine returned to their nest after the first foraging trip, and seven of these returned without passing through any of the other shelters. This is significantly better (χ^2 -test, $P = 0.01$) than would be expected if the spiders remained at the first shelter they found by chance. When the spiders had their PM eyes covered by black paint, only three out of ten spiders returned to the original shelter. Unpainted spiders performed equally badly (three out of ten) when the polarized illumination was replaced by unpolarized light. Neither of the latter experiments is significantly different from the 25% success rate expected from a random search. We can thus be confident that spiders use polarization cues seen by the PM eyes to find their way home.

A compass organ, similar to that of *D. cupreus*, can be inferred from the structure and configuration of the PM eyes of numerous species from several spider families within the superfamily Gnaphosoidea¹⁵. The Australian species *Lampona cylindrata* has PM eyes that are practically identical to those of *C. cupreus*²¹, and they behave as polarizers in the same way as in Fig. 1. A preliminary survey of secondary eyes across spider taxa shows that one or several of the key features reported here (absent or poor focusing, a dominant class of uniformly oriented receptors and a polarized tapetal reflection) are almost universally present in species with canoe-type reflecting tapeta, thus offering a possible explanation of

this common but enigmatic type of spider eye. Because the compass organ reported here appears to handle no other visual tasks, it also offers an interesting model for studies of the neural processing underlying polarization navigation. □

Methods

Spiders

D. cupreus were collected in the vicinity of Lund, Sweden. We used adults (10–17 mm body length) of both sexes. Comparative investigations were performed on a range of spider species collected in Sweden, Australia and New Zealand. Descriptions below refer to investigations on *D. cupreus*. Sections for light and electron microscopy were prepared using conventional techniques²².

Polarization experiments

The photographs in Fig. 1 were taken using an optical apparatus described previously²³, fitted with a polarizer in the illumination beam path but not in the observation beam path. The light source was a 75W xenon arc lamp. The degree of polarization reflection was measured with a photomultiplier attached to the apparatus, which integrated over the entire eye. We searched for polarizing eye structures using eyes teased apart in physiological saline and inspected fresh with incident and transmitted light polarization microscopy.

Focal length measurements

Pieces of the dorsal exoskeleton containing the clear sections situated above the PM retinas were rinsed free of tissue and placed in a water drop hanging from the underside of a suspended coverslip. The external corneal surface was in air and the internal surface in water¹⁶. Objects (large letters and striped patterns) were placed 10 cm away on the air side and a microscope was used to inspect any images formed on the water side.

Electrophysiology

Intact animals were immobilized with wax and a small hole was cut at the edge of the PM eye. Intracellular glass electrodes were inserted through the hole and aimed at the retina. The few successful recordings gave saturating responses of 45–50 mV. For each cell, a response–intensity curve was recorded at log(intensity) increments of 0.2, with light polarized parallel to the long axis of the eye. A second curve was then recorded with light polarized in the perpendicular direction. The intensity shift between the two curves, caused by the different planes of light polarization, was used to compute the polarization sensitivity⁹. We recorded coarse spectral sensitivities across the range 300–700 nm at 50 nm increments. The spectral sensitivity was also measured with electroretinography (extracellular mass recordings of the entire retina), using low-resistance glass electrodes inserted just under the cornea (providing maximum responses of about 2 mV).

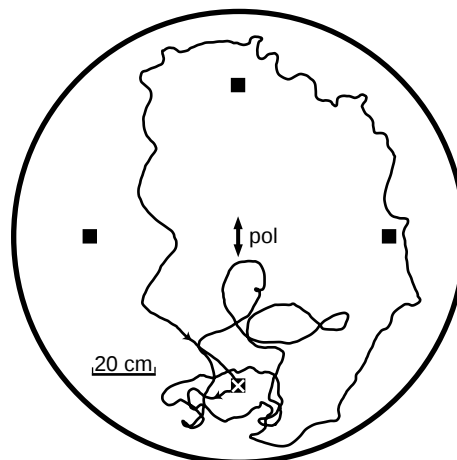
Model eye experiments

A 1-cm² photodiode was fitted with a band-pass interference filter transmitting light at 350–450 nm, and a motor-driven, rotating ultraviolet-blue polarizer (Polaroid, HNP'B). The signal modulation, caused by rotating the polarizer, gave measurements of the available polarization cues. The device was mounted on the roof of the Zoology building in Lund, and shielded to integrate evenly across a 128° field of the sky, centred on the zenith. Recordings were run continuously over 24-h periods of clear or lightly clouded skies.

Activity measurements

Eight spiders were placed in individual 183-mm Petri dishes standing on white paper with sixteen equally sized sectors marked out. A shelter (3.5-cm diameter dark plastic lid, supported on one side) was placed in each dish above one of the sectors. The spiders were habituated for two days under a natural illumination cycle. On the third day, when all

Figure 4 Layout of the arena used for navigation experiments. Squares represent four symmetrically placed shelters. The excursion trail is drawn for one of the experiments where the spider successfully exploited polarization cues, seen by the secondary eyes, to return to the shelter under which a silk nest previously had been produced (white cross). Pol, direction of polarization of light.



spiders had made silk nests under their shelters, the occupied sector was noted every 15 min for 24-h periods. Activity was defined as a change of sector. As the spiders were not fed during the experiment, it is possible that the activity period was longer than if prey had been found.

Navigation experiments

Individual spiders were placed in a circular arena (1.5 m in diameter) with four symmetrically placed shelters as shown in Fig. 4, and the movements of the spiders were recorded on video from above. A lamp placed 1.5 m above the centre of the arena provided dim light corresponding to the natural intensity about 1 h after sunset. The spectral composition of the artificial sky stimulus was that of a filament bulb at 4,000 K. The lamp was fitted with a removable polarizing sheath. In addition to the artificial light from the lamp, weak indirect (unpolarized) daylight from a distant window produced a faint intensity gradient across the ceiling (providing cues similar to the real sky and resolving the 180° ambiguity of a pure polarization compass⁶). After 24 h of habituation, the spiders had selected one of the shelters and lined it with a silk nest. At the time of behavioural recordings, the natural ceiling light was weaker than the artificial light. The excursion paths made by the spiders were traced out from the video recording. Excursions during which the spiders either moved along the wall of the arena for more than half the circumference, or moved less than half way towards a neighbouring nest were ignored. When a spider entered and remained in a shelter for more than 2 min, the excursion was considered to have ended. The rate of return to the home shelter was investigated under three different experimental conditions: with the polarizing sheath in place; with the sheath removed; and with the sheath in place but with the secondary eyes covered with opaque paint (we left the principal eyes uncovered, but painted all three pairs of secondary eyes, not just the PM eyes, because the other secondary eyes somewhat resemble the PM eyes and may provide some input to the polarization compass). Ten spiders were used to obtain ten complete excursion recordings from each of the three types of experiment.

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Supplementary Information is available at Nature's World-Wide Web site (<http://www.nature.com>) or as a paper copy from the London editorial office of Nature.

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Dorsoventral lineage restriction in wing imaginal discs requires Notch

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The formation of boundaries that prevent the intermixing of cells is an important developmental patterning mechanism. The compartmental lineage restrictions that appear in the developing imaginal discs of *Drosophila* are striking examples of such boundaries¹. However, little is known about the cellular mechanism underlying compartmental lineage restrictions. The dorsoventral (D/V) lineage restriction that arises late in the developing wing imaginal disc requires the dorsal expression of the transcription factor Apterous and it has been hypothesized that *apterous* (*ap*) maintains compartmentalization by directly regulating the expression of molecules that modify cell adhesion or affinity². However, *ap* expression also regulates signalling between dorsal and ventral compartments, resulting in high levels of Notch signalling at the D/V boundary^{3–17}. Here we show that the formation of Notch-dependent boundary cells is required for the D/V lineage restriction.

The *ap* expression domain is congruent with the dorsal lineage compartment^{3,18} (Fig. 1a). High levels of Notch signalling at the *ap* boundary induce the expression of 'boundary-specific' genes, such as *cut* and *wingless* (*wg*)^{4–13}. This Notch signalling is thought to be triggered by *ap* regulating the compartment-specific expression of the Notch ligands Serrate^{6,7} and Delta^{10,11}, and by regulating each compartment's sensitivity to these ligands by inducing the dorsal expression of Fringe^{14–17}. Serrate in the dorsal compartment signals mostly to adjacent ventral cells that lack Fringe, and Delta in the ventral compartment signals mostly to adjacent dorsal cells expressing Fringe (Fig. 1b). Late in development, high levels of Serrate and Delta are expressed in cells flanking the boundary cells, maintaining Notch activity in the boundary cells but blocking it in the flanking cells^{12,13} (Fig. 1c). The activity of Notch is further heightened by a positive feedback loop that increases the expression of Serrate and Delta in response to Notch signalling^{13,15}. Removing Notch from cells on one side of the Apterous boundary not only disrupts Notch-dependent gene expression within the mutant cells, but also variably disrupts Notch-dependent gene expression in wild-type cells on the other side of the boundary^{4,12} (Fig. 2f).

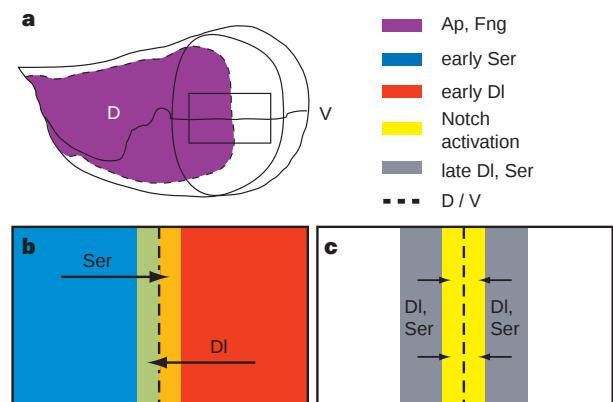


Figure 1 Notch signalling along the dorsoventral (D/V) compartment boundary of the wing imaginal disc. In this and subsequent figures, anterior is up and ventral is right; Ap, Apterous; DI, Delta; Fng, Fringe; N, Notch; Ser, Serrate. **a**, Late third-instar wing disc. **b, c**, Signalling (see text). **b**, Early stages; **c**, later stages.

To test the role of Notch signalling in maintaining the D/V boundary, we generated mitotic recombinant clones in larvae heterozygous for N^{55e11} , a null allele of *Notch*¹⁹. Mitotic recombination generates two cells, one homozygous for N^- , identifiable by the absence of the *marker* gene, and the other homozygous for the *marker* and identifiable by its stronger staining. We used the position of the neighbouring homozygous *marker* clone to identify the compartmental origin of the mutant clone independent of its final position. The dorsal-specific expression of *ap* and the largely ventral expression of PS2 integrin²⁰ allowed us to confirm the compartmental origin of clones and resolve ambiguous cases in which a clone was associated with several homozygous *marker* clones. We marked the site of the D/V boundary outside the clone by following *ap* expression, and in some cases by following the boundary-specific expression of *cut* and *wg*.

The majority of N^- clones that were in contact with the *ap* boundary disrupted the D/V lineage restriction (Table 1, Fig. 2). In the most extreme class, N^- clones straddled or crossed the normal site of the D/V boundary from either the dorsal or ventral side (Fig. 2b, c, e, f), with most or all of the cells in such clones being in the wrong compartment (Fig. 2c, e, f). In some cases the distortions

were milder ('bulging' clones; Fig. 2a, d) and some clones approximately respected the boundary. Disruptions were not always limited to the mutant clone as in some cases the cells in the compartment adjacent to the N^- clone pushed into the compartment containing the N^- clone, distorting the D/V boundary from the other side (Fig. 2d). The severity of the abnormalities was especially pronounced in clones generated at or just after the formation of the D/V restriction (72 to 60 h before pupariation; see Fig. 2 legend), but could still be observed in smaller clones generated at later stages (60 to 48 h before pupariation; Table 1).

Disruption the D/V lineage restriction by N^- clones was not brought about by changing *ap* expression (Fig. 2) or the expression of the *ap*-regulated PS2 integrin (not shown). Many clones could be assigned an unambiguous compartmental origin by their association with a single homozygous *marker* clone. N^- cells of unambiguously dorsal origin that crossed into ventral territory autonomously maintained *ap* expression (Fig. 2a, b). N^- cells of unambiguously ventral origin that crossed into dorsal territory lacked *ap* expression (Fig. 2d–f). Moreover, N^- clones that were distant from the D/V boundary never gained or lost *ap* expression (Fig. 2b, d, e).

It is unlikely the phenotypes of straddling and crossing N^- clones can be accounted for by general differences in the survival or growth of N^- clones. Cell numbers and clone frequencies are lower for N^- than for equivalent wild-type clones²¹, but it is unlikely that lower cell numbers within a clone would cause it to push into an adjacent compartment. Nor do N^- clones generally disrupt lineage restric-

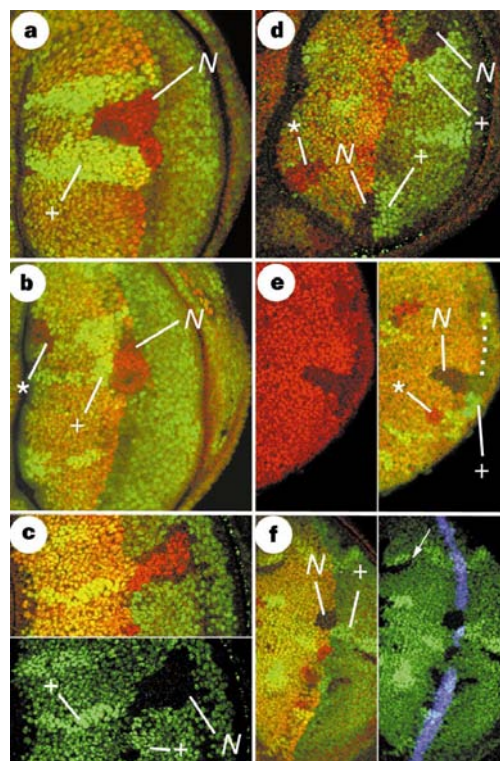


Figure 2 Late third-instar wing discs containing N^- clones (N^-) generated at 72–60 h before pupariation, marked by the absence of a *marker* gene (green). Increased *marker* expression marks homozygous *marker* clones (+). Asterisks mark N^- clones distant from the D/V that retain their normal state of *ap* expression (b, d, e). Anti-*Ap* staining, red; anti-*Wg*, blue. **a–c**, Clones of dorsal origin showing *ap* expression. **a**, A 'bulging' N^- clone. **b**, A 'straddling' N^- clone. This γ -ray-induced clone is of unambiguous dorsal origin, as its associated + clone is dorsal. There is *ap* expression throughout the clone. **c**, A 'crossing' N^- clone entirely in ventral territory. Most but not all of the clone expresses *ap* and, as it is associated with dorsal and ventral + clones, it is of mixed origin. The fusion of dorsal and ventral N^- clones was common using FLP/FRT clone induction because of the high density of clones. **d–f**, N^- clones of ventral origin, showing no *ap* expression. **d**, A bulging posterior N^- clone. An anterior N^- clone does not cross the D/V boundary, but adjacent cells in the opposite compartment push ventrally, distorting the boundary from the dorsal side. **e**, A crossing N^- clone. The disc is partly everted, obscuring much of the ventral compartment; the dotted line marks the D/V. **f**, A crossing N^- clone that induces the loss of *wg* expression along both sides of the *ap* boundary. The arrow marks a fold in the disc.

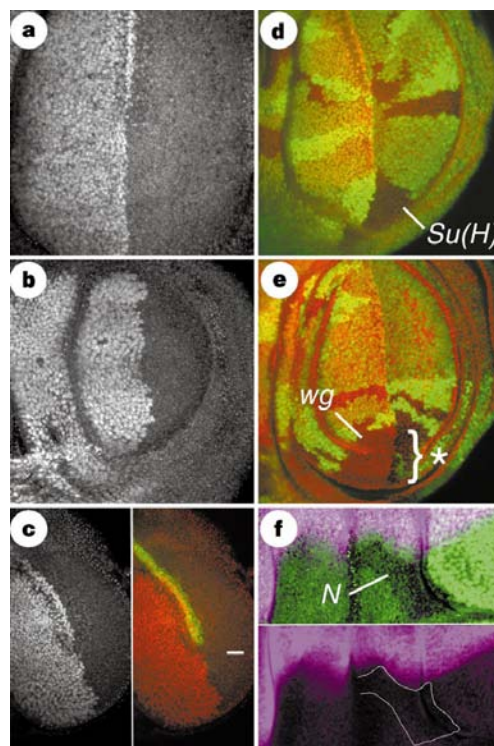


Figure 3 The role of signalling at compartment boundaries. **a–c**, Reductions in Notch function disrupt the *ap* expression boundary. **a**, **b**, Homozygous N^{55e11} discs stained with anti-*Ap*. **a**, Disc reared at the permissive temperature. **b**, Disc shifted to the non-permissive temperature for 48 h before pupariation. The reduced size of the wing pouch is characteristic of reduced Notch function⁴. **c**, Disc expressing dominant-negative Notch in the posterior compartment (*en-GAL4; UAS-ECN*, grown at 18 °C and shifted to 30 °C for 72 h before pupariation). Dash indicates A/P. Right, anti-*Ap* staining. Left, double image with anti-*Ap* (red) and anti-*Wg* (green) staining. **d**, A large *Su(H)* clone obeys the D/V lineage restriction. **e**, A *wg*⁻ clone, generated before the formation of the D/V lineage restriction and straddling the D/V, does not disrupt the *ap* boundary (bracketed region). **f**, A posterior N^- clone respects the A/P boundary. The anterior compartment is marked by anti-Cubitus interruptus (pink).

tions, as they obeyed the anterior–posterior (A/P) compartmental lineage restriction (7 anterior and 3 posterior clones; Fig. 3f), in agreement with previous studies²¹.

Disc-wide reductions in Notch activity also disrupted the D/V boundary. Normally, the boundary of *ap* expression is precisely localized and relatively smooth, indicating a difference in affinity between dorsal and ventral cells (Fig. 3a). Both these features were lost after Notch function was removed for the last 48 or 72 h of larval development using *N^{ts1}*, a temperature-sensitive hypomorphic allele²². This perturbation disrupted the normally straight boundary of *ap* expression along the D/V boundary, and led to extensive interdigitation between cells expressing and not expressing *ap*, giving the boundary a ragged appearance (Fig. 3b). Similar disruption was seen after a 24 h shift using the stronger *N^{ts1}/N^{55c11}* genotype (not shown). It is unlikely that these phenotypes were caused by cell death, as only limited cell death is observed along the wing margins of *N^{ts1}/N^{55c11}* after 36 h shifts⁴. Expressing a dominant-negative form of the Notch receptor which retains the extracellular and transmembrane portions of Notch but lacks the intracellular domain²³, also induced ragged, irregular *ap* boundaries. Disruptions were observed after expression of the dominant-negative Notch in either the posterior compartment (Fig. 3c) or along the D/V boundary (not shown).

The Su(H) transcription factor is thought to mediate most but not all of the signalling downstream of the Notch receptor²⁴. However, clones homozygous for the strongest available *Su(H)* allele, *Su(H)^{SF8}*, did not reliably disrupt the D/V boundary. Clones induced after the formation of the D/V restriction obeyed the restriction (24 clones, Fig. 3d), whereas clones induced before its formation and that straddled the D/V boundary did not disrupt (9 clones) or only slightly disrupted (3 clones) the shape of the *ap* boundary (not shown). This indicates that the requirement of Notch in maintaining the lineage restriction may be largely independent of *Su(H)*.

This result is surprising, as the expression of Notch-dependent genes at the D/V boundary is lost from identical *Su(H)⁻* clones^{9,25}. However, the available *Su(H)* alleles are not complete protein

Table 1 Behaviour of *N⁻* clones at the D/V boundary

	Number of clones, dorsal origin/ventral origin			
	72–60 h BP			48 h BP
	Total	Loss of anti-Wg staining*		
Bilateral		Unilateral		
Clones that respect D/V boundary†	39/10	1	15	2/1
Clones that bulge‡	17/11	6	0	1/2
Clones that are pushed§	17/32	11	3	1/1
Clones that straddle or cross (unambiguous origin¶)	47/23 (17/13)	7 (2)	0 (0)	1/4 (1/1)
Less than half of clone crosses	4/0 (1/0)			
About half of clone crosses	17/5 (7/3)			
More than half of clone crosses	14/8 (6/4)			
Entire clone crosses	12/10 (3/6)			

* A subset of the clones in 'Total' were stained with anti-Wg. Unilateral loss was limited to the clone; bilateral loss included cells opposite the clone.

† Clones defined the lineage restriction for at least four cell diameters.

‡ Clones extending two cell diameters into adjacent dorsal or ventral territory.

§ Clones that induce cells in the compartment opposite the clone to extend two or more cell diameters into the compartment containing the clone.

|| Clones extending more than two cell diameters into adjacent dorsal or ventral territory.

¶ Numbers in parentheses indicate the subset of straddling or crossing clones made up wholly of cells with an unambiguous compartmental origin.

BP, before pupariation.

nulls²⁶, so it is possible that the clones retain low levels of residual Su(H)-mediated Notch activity. Alternatively, Notch might act directly as an adhesion molecule: *in vitro*, Notch-expressing cells adhere to those expressing *Delta*²⁷. Notch might mediate different forms or amounts of adhesion depending on the combination of *Delta*, *Serrate* or *fringe* expressed in each compartment, with *N⁻* lacking an affinity for either compartment and taking an ambiguous position. However, expression of the extracellular, dominant-negative form of Notch might be expected to increase Notch-based adhesion, yet it induces a ragged *ap* boundary (Fig. 3c). Moreover, clones homozygous for *N^{co}*, which encodes a form of Notch lacking only the intracellular portion of the molecule²⁸, still disrupt the D/V lineage restriction (7 out of 8 clones; not shown).

An important consequence of the loss of the Notch activity at the D/V boundary is the loss of *wg* expression. The Wg morphogen affects growth and gene expression in the wing¹, so it could be required to maintain the D/V restriction. However, removing Wg from both sides of the D/V boundary had no effect on the shape of the *ap* boundary (14 clones; Fig. 3e). The disruptions caused by the loss of Notch activity are therefore either mediated directly by the loss of Notch activity, or indirectly by some as yet unknown factor.

Our results indicate that some or all of the requirement for *ap* in establishing the D/V lineage restriction may be mediated by its effects on Notch signalling. As at the A/P restriction²⁹, complex models based both on cell affinities and patterned growth can be proposed, and it remains possible that Apterous drives affinity in a manner independent of signalling. However, a simple affinity-based model, in which *ap* changes the response to Notch signalling, is sufficient to explain our results (Fig. 4). We suggest that there are three different states of affinity: a dorsal, boundary-specific affinity state 'D', specified by the activity of *ap* and high levels of Notch signalling; a ventral, boundary-specific affinity state 'V', specified by high levels of Notch signalling alone, and a default affinity state 'X'. Cells can move within each compartment because they can all receive the Notch signal, and thus acquire state D or V if they are near the margin. However, the clonal inheritance of *ap* expression prevents cells from switching between states D and V: as long as Notch activity remains high, cells will not cross the D/V boundary.

N⁻ clones that lie on one side of the D/V boundary variably disrupt Notch signalling both within the clone and in adjacent cells in the other compartment^{4,12}. In our model, such clones will result in the loss of cell state D or V within the clone, and V or D in cells opposite the clone (Fig. 4). Cells both within and adjacent to the *N⁻* clone acquire cell-state X, and either the clone can move into the

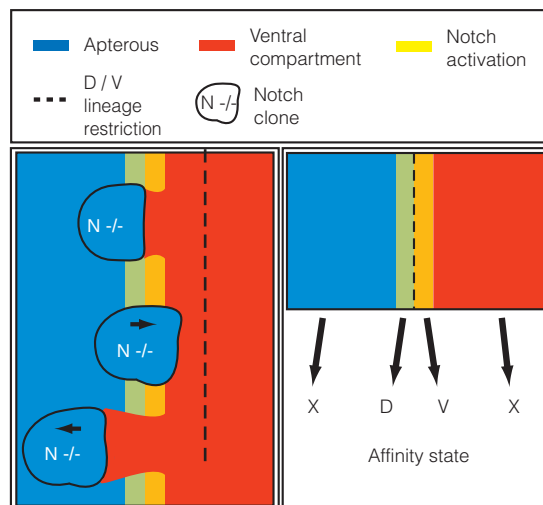


Figure 4 Model for the maintenance of the D/V lineage restriction. Left, behaviour of *N⁻* clones. Right, the model. A high level of Notch (N) signalling specifies cell-affinity state D or V in the presence or absence of *ap* expression, respectively; all other cells have affinity-state X. The model requires the loss of both D and V to induce crossing; see text. The model can also account for the ability of dorsal *ap⁻* clones to cross the D/V². Dorsal *ap⁻* clones at the D/V boundary disrupt Notch signalling on the ventral edge of the clone, but re-establish signalling at the ectopic *ap* boundary^{3,5}. The tendency to minimize contacts between cells in states D and V, coupled with the affinity of cells with cell-state X, will straighten the boundary and move the *ap⁻* clone into ventral territory.

adjacent compartment or the cells opposite the clone can push into the compartment containing the clone (Fig. 4). The model further predicts that those N^- clones that do not disrupt Notch activity in the compartment opposite the clone will not disrupt the lineage boundary, as cells across the boundary from the clone will retain cell state D or V. Indeed, we observed a near-perfect correlation between the loss of Notch-dependent *wg* expression opposite the clone and the ability of the clone to disrupt the D/V boundary (Fig. 2f; Table 1). Our model also predicts extensive disruption of the *ap* boundary when dorsal and ventral N^- clones meet, as was often observed (Fig. 2c).

Our results showing a role for Notch signalling in the maintenance of the D/V lineage restriction mirror recent analyses of the A/P lineage restriction; Hedgehog signalling from the posterior to the anterior, driven by the posterior selectors *engrailed* and *invected*, is critical for the affinity of anterior cells^{29,30}. Thus, the dual requirement for autonomous, selector-like activity and non-autonomous, selector-driven signalling may be a general feature in the maintenance of lineage compartments. □

Methods

Mitotic recombination.

We generated *Su(H)^{SF8}*, *wg^{CK4}*, *N^{Co}* (provided by G. Struhl) and most *N^{55el1}* clones using FRT/FLP mediated mitotic recombination^{4,12}. To induce less crowded *N^{55el1}* clones we used γ -rays (2.5–4 krad). We timed clone induction by picking white prepupae at set times after heat shock or irradiation. Clones were marked by the absence of the ubiquitously expressed π M or *WGL296* markers.

Timing of D/V boundary formation.

Marked clones were generated using FRT/FLP (π M *FRT^{18A}*; *FLPase* $\times$ *white⁻ FRT^{18A}*) at set times before pupariation and scored if they were in contact with the D/V boundary. The number of *white⁻* clones separated from their associated π M clone by the D/V was 1 out of 7 at 72 h, 3 out of 19 at 60 h and 0 out of 12 at 50 h before pupariation.

Reducing Notch activity.

N^{ts} experiments were performed as described previously^{4,12}. We drove expression of dominant-negative Notch with the UAS–GAL4 system¹, using *UAS-ECN* (provided by S. Artavanis-Tsakonas), *en-GAL4* (posterior) and *vg^{intronic2}-GAL4* (boundary; provided by S. Carroll). We used the cold sensitivity of GAL4 to minimize the effects of *en-GAL4* expression on early stages of development, rearing larvae at 18°C and shifting to 30°C for 24, 48, 72 or 96 h before pupariation.

Immunohistology.

We performed immunohistology as described^{4,12,18,29}, except in some cases we used guinea-pig anti-Ap overnight at 1/500 (provided by J. Botas).

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Fringe-dependent separation of dorsal and ventral cells in the *Drosophila* wing

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The separation of cells into populations that do not intermix, termed compartments, is a fundamental organizing principle during development^{1–5}. Dorsal–ventral compartmentalization of the *Drosophila* wing is regulated downstream of the *apterous* (*ap*) gene, which encodes a transcription factor that specifies dorsal wing fate^{6–8}. *fringe* (*fng*) is normally expressed by dorsal cells downstream of *ap*⁹; here we show that *fng* plays a key role in dorsal–ventral compartmentalization. Loss of *fng* function causes dorsal cells to violate the compartment boundary, and ectopic expression of the Fng protein causes ventral cells to violate the

compartment boundary. *Fng* modulates signalling through the Notch receptor^{10,11}. Notch and its ligands are essential for formation of the dorsal–ventral compartment border, and repositioning the stripe of Notch activation that is normally established there appears to reposition the compartment border. However, activation of Notch does not itself confer either dorsal or ventral cell location, and *fng* can influence compartmentalization even within regions of ubiquitous Notch activation. Our results indicate that the primary mechanism by which *fng* establishes a compartment border is by positioning a stripe of Notch activation, but also that *fng* may exert additional influences on compartmentalization.

To investigate the involvement of *fng* in dorsal–ventral (D–V) compartmentalization, we analysed clones of cells that were mutant for *fng* in developing wing imaginal discs. These clones were generated by mitotic recombination, and marked by loss of expression of a Myc-epitope-containing protein¹². Mitotic recombination also generates wild-type twin clones, which can be identified by elevated Myc staining. To assess the dorsal or ventral origin of cells, we assayed *ap* expression with a reporter gene, *ap-lacZ*⁶, which stably marks clones of dorsal origin even if they are mutant for *ap* and cross into the ventral compartment⁸.

fng mutant clones that originate in the dorsal compartment fail to respect the D–V border (Fig. 1a–d, Table 1), as shown by the appearance of *ap*-expressing *fng* mutant clones in the ventral compartment. Their wild-type twins remain in the dorsal compartment. *fng* mutant ‘cross-over’ clones often form relatively straight borders with wild-type dorsal cells; that is, they often appear to respect a D–V border from the ventral side (Fig. 1a–d). This repositioned border can integrate into the normal D–V border made by flanking wild-type cells, as visualized by Wingless (Wg) expression (Fig. 1a, b, d), which is positioned along the D–V border by the influence of *Fng* on Notch activation^{9,13–15}. In some cases,

however, *fng* mutant clones appear to straddle the D–V border (Fig. 1c). Additionally, ventral *fng* mutant clones of dorsal origin tend to remain near the D–V boundary (Fig. 1a–d). The last two observations may reflect *fng*-independent influences on D–V compartmentalization, but are also consistent with the possibility that *Fng* perdures, or that temporal or spatial constraints limit the extension of clones of cells into the ventral compartment.

The appearance of *ap*-expressing cells in the ventral compartment does not reflect an influence of *fng* on *ap* expression. Ventral *fng* mutant clones of ventral origin occur at the same frequency as their wild-type twins, do not ectopically express *ap-lacZ* and can define the D–V border from the ventral side (Fig. 1e, Table 1). Dorsal *fng* mutant clones that do not touch the D–V border can be trapped in the dorsal compartment, and they too express *ap* normally (Fig. 1f).

To determine whether *Fng* is not only necessary for normal dorsal cell location, but also sufficient to alter the location of ventral cells, we used the Flip-out Gal4 technique to produce clones of cells ectopically expressing *Fng*^{16–18}. These clones were marked by co-expression of a *UAS-GFP* transgene, and their influence on D–V compartmentalization was monitored with *ap-lacZ* expression and compared with that of control clones (Table 2, Fig. 2).

The behaviour of Flip-out *Fng* clones mirrors that of *fng* mutant clones. We observed no ventral Flip-out *Fng* clones that respected the D–V border from the ventral side (Table 2). Instead, these

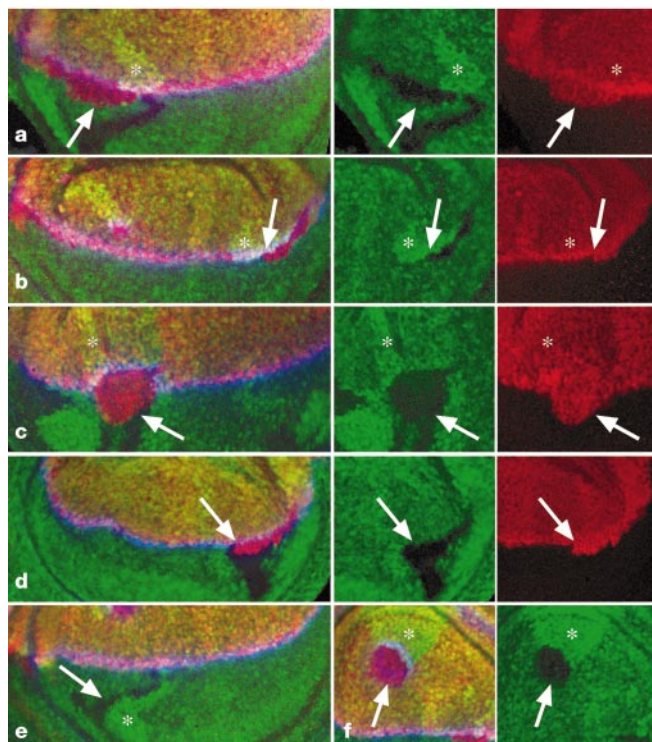


Figure 1 Loss of *fng* activity causes dorsal cells to violate the compartment boundary. All figures show late third instar wing imaginal discs, with dorsal up. **a–f**, *fng* mutant clones stained for π -Myc (green), Wg (blue) and *ap-lacZ* (red). Green and red panels to the right show individual stains for highlighted clones. Arrows, mutant clones; asterisks, wild-type twins. **a–d**, *fng* mutant clones in which dorsal cells are located ventrally. **e**, Ventral *fng* mutant clone. **f**, Dorsal *fng* mutant clone that does not touch the border.

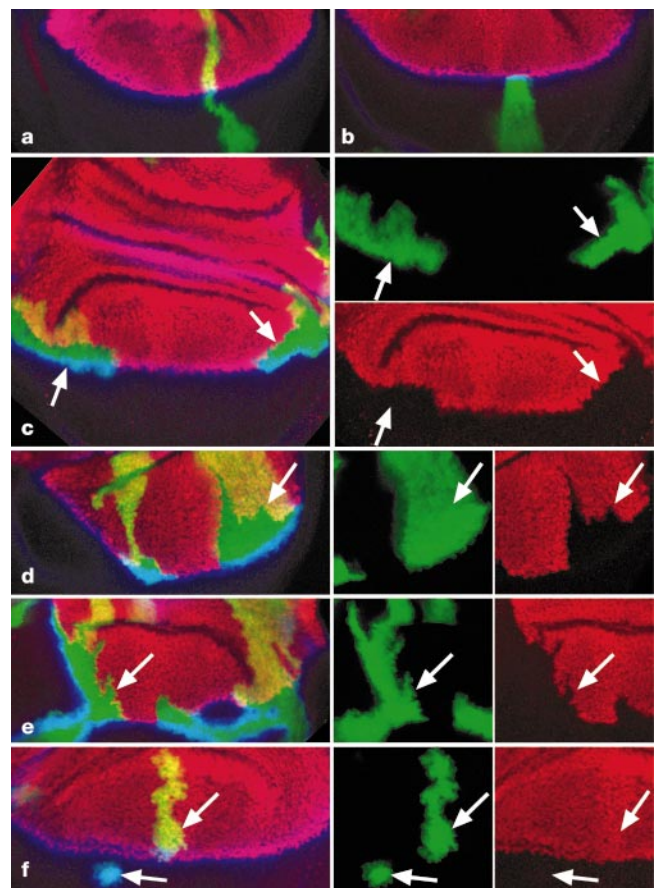


Figure 2 Ectopic expression of *Fng* causes ventral cells to violate the compartment border. Discs were examined for expression of GFP (green), Wg (blue) and *ap-lacZ* (red). **a**, Mixed-identity control clone. **b**, Ventral control clone that touches and respects the border. **c–f**, Flip-out *Fng* clones. Green and red panels to the right show individual stains for highlighted clones. **c**, Clones of ventral identity that are found dorsally. **d**, **e**, Clones of mixed identity in which ventral identity cells extend into and intermix with dorsal cells, while the *Fng* expression border with ventral cells forms an extended straight line. **f**, A ventral Flip-out *Fng* clone that does not touch the D–V border (bottom arrow), and a dorsal Flip-out *Fng* clone that does not influence *ap* expression or clone behaviour (top arrow).

ventral Flip-out Fng clones are found in the dorsal compartment, as indicated by cells that are located dorsally and do not express *ap-lacZ* (Table 2, Fig. 2c–e). Cross-over clones that are entirely ventral in origin tend to be found near the D–V border (Fig. 2c); however, the ventral (*ap*-non-expressing) cells of clones that are generated

early enough in development to be of mixed identity can be found far from the D–V border (Fig. 2d, e). From this, we infer that the preference of cross-over clones to remain near the border reflects their history, rather than some remaining affinity for ventral compartment cells. Flip-out Fng clones of ventral origin that are located dorsally intermix with surrounding dorsal cells, especially when they are induced early, and appear to define a new D–V border with non-Fng-expressing cells (Fig. 2c–e). Flip-out Fng clones that do not come in contact with the D–V border can be trapped in the ventral compartment (Fig. 2f). Dorsal Flip-out Fng clones of dorsal origin occur at the same frequency as control clones, intermix normally with surrounding cells, do not influence *ap* expression, and can respect the D–V border from the dorsal side (Fig. 2f, Table 2).

Fng influences signalling between dorsal and ventral cells by modulating the ability of the Notch ligands Serrate (Ser) and Delta (Dl) to activate the Notch receptor^{10,11,14}. To assess the role of Notch and its ligands in D–V compartmentalization, we examined clones of cells that were mutant for *Notch* or doubly mutant for *Dl* and *Ser*. In many cases, *Notch* (11 out of 14) or *Dl Ser* (47 out of 84) null mutant clones that touch the D–V compartment border fail

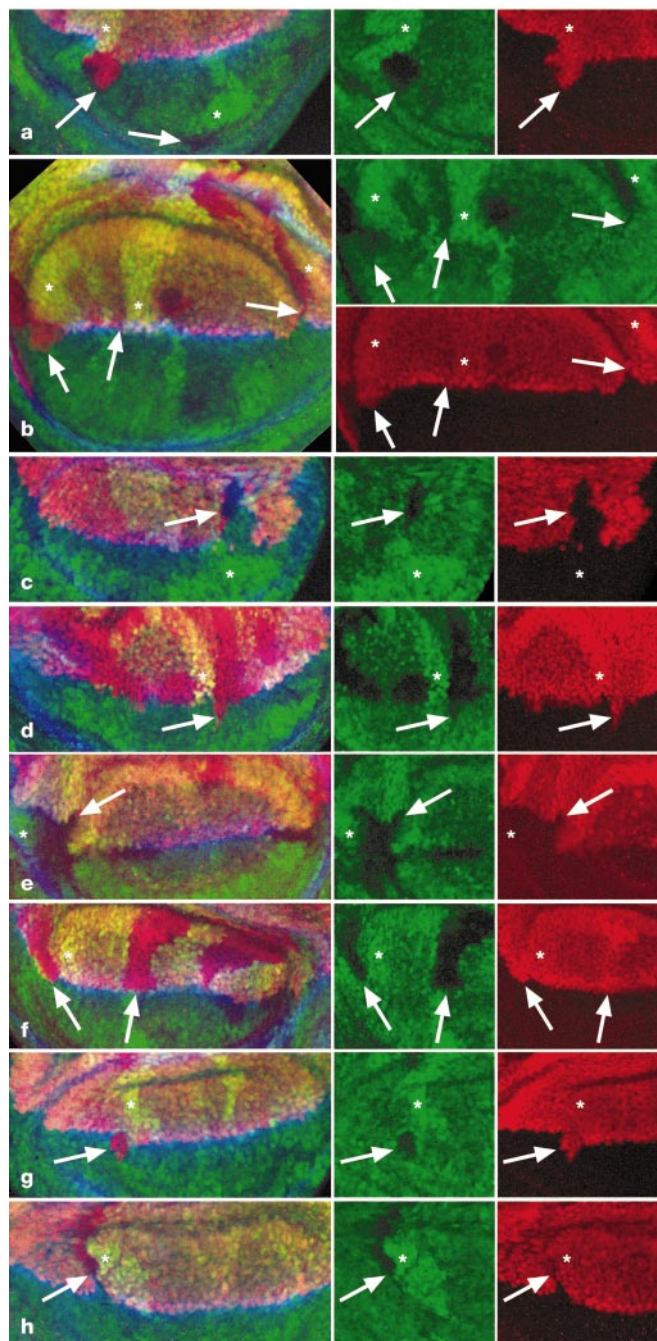


Figure 3 Notch signalling is required for D–V compartmentalization. Discs were stained for π -Myc (green), Wg (blue) and *ap-lacZ* (red). Green and red panels to the right show individual stains for highlighted clones. **a–c**, *Notch*^{55e11} clones. **a**, Left arrow shows violation by dorsal cells of a mixed-identity clone, right arrow shows a ventral clone with no effect on *ap* expression. **b**, Left arrow shows violation by a dorsal clone, middle arrow shows a clone that does not eliminate Wg and respects the border, right arrow shows a dorsal clone that induced a non-autonomous violation. **c**, Ventral clone that violated the compartment border. **d–f**, *Df^{ev10}Ser^{RX106}* clones. **d**, Dorsal clone that violates the border. **e**, Ventral clone that violates the border. **f**, Left arrow shows violation by a dorsal clone, right arrow shows a dorsal clone that does not eliminate Wg and respects the border. **g–h**, *Notch*^{CO} clones. **g**, Dorsal clone that violates the border. **h**, Dorsal clone that induces a non-autonomous violation.

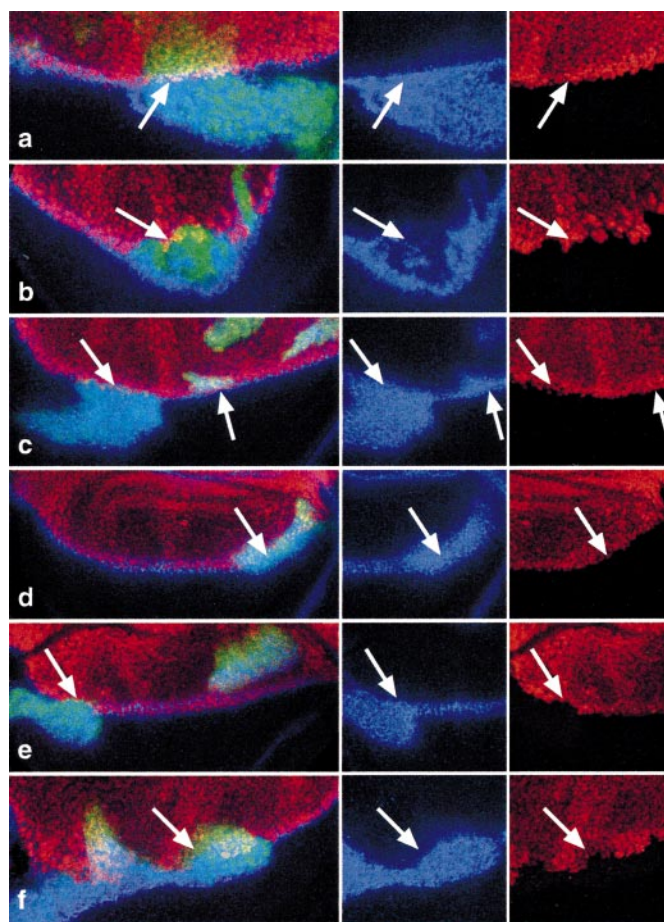


Figure 4 Influence of ectopic Ser, activated Notch, and activated Notch plus Fng on D–V compartmentalization. Discs were examined for expression of GFP (green), Wg (blue) and *ap-lacZ* (red). Blue and red panels to the right show individual stains for highlighted clones. **a, b**, Flip-out Ser clones. **a**, A clone of mixed D–V identity that does not inhibit endogenous Notch activation and does not disturb the compartment border. **b**, A clone that inhibits endogenous Notch activation and disturbs compartmentalization. **c, d**, Flip-out activated-Notch clones. **c**, Left arrow shows a clone that respects the D–V border from the ventral side, right arrow shows a clone that respects the D–V border from the dorsal side. **d**, A clone of mixed D–V identity, the edge of *ap* expression within the clone is undisturbed. **e, f**, Flip-out activated-Notch plus Fng clones. Slight disturbances in D–V compartmentalization are observed.

Table 1 Influence of *fng* mutant clones on compartmental location

<i>fng</i> mutant clones				Wild-type twins			
Identity	No.	Location	No.	Identity	No.	Location	No.
Dorsal	124	D—internal D—border Ventral	89 1 34	Dorsal	119	D—internal	97
						D—border	22
						Ventral	0
				Ventral	3	Ventral	3
Mixed*	9	Dorsal Ventral	2 7	Mixed	2	Dorsal	2
						Ventral	0
						Dorsal	0
				Ventral	73	V—internal V—border Dorsal	54 19 0
Ventral	78	V—internal V—border Dorsal	38 40 0	Dorsal	2	Dorsal	2
						Ventral	0
						Dorsal	0
				Mixed	3	Dorsal Ventral	3 0
Mixed*	9	Dorsal Ventral	2 7	Dorsal	1	Dorsal	1
						Ventral	0
						Dorsal	0
				Ventral	3	Ventral Dorsal	3 0
Mixed*	9	Dorsal Ventral	2 7	Mixed*	5	Dorsal	5
						Ventral	0
						Dorsal	0
						Ventral	0

Identity was defined by *ap-lacZ* expression. In agreement with previous studies^{8,28}, we found that 94% (73/78) of the twins of ventrally located clones that were induced 52–72 h after egg laying were also located ventrally. In addition, 95% (192 twins/202 dorsal or ventral *fng*^{CO} clones) of wild-type twins were of the same identity as the mutant clone, and 96% (202/211) of all clones were strictly dorsal or ventral in identity. The locations of wild-type twins, and of ventral *fng* clones, always match their identity. By contrast, 97% (34/35) of dorsal *fng* clones that came in contact with the D–V border violated it. Locations were scored as ventral if any part of the clone appeared on the ventral side of the D–V border; in 72% of cases, this cross-over was judged to be complete.

* Among clones with mixed identity, only *ap*-expressing cells were scored.

to respect it, as judged by alterations in the location of *ap-lacZ*-expressing cells (Fig. 3a–f). These changes do not reflect an influence of Notch signalling on *ap* expression, as *ap-lacZ* is unaffected in *Notch* or *Dl Ser* clones that do not touch the D–V border (Fig. 3a; and data not shown).

Notch and its ligands can act as cell-adhesion molecules in cultured *Drosophila* cells¹⁹. We analysed several classes of experiments to assess the relative contributions of Notch activation versus participation in adhesive interactions to D–V compartmentalization. In cultured cells, Notch-mediated adhesion requires only the extracellular domain of Notch²⁰. *Notch*^{CO} encodes a protein with an intact, expressed (not shown) extracellular domain, but a truncated, non-functional intracellular domain²¹. However, *Notch*^{CO} mutant clones disrupt D–V compartmentalization just like clones that are mutant for a protein null allele, *Notch*^{55e11} (Fig. 3g, h; 9 out of 10 *Notch*^{CO} clones that touched the D–V border altered the location of *ap-lacZ*-expressing cells). Thus, the extracellular domain of Notch is not sufficient for D–V compartmentalization.

In the wild type, Notch is activated along both sides of the compartment border, and Notch activation is maintained there in part by a feedback loop between dorsal and ventral cells¹⁴. Consequently, many *Notch* or *Dl Ser* mutant clones that contact the D–V border from only one compartment also disrupt Notch activation in the opposite compartment^{15,22}. However, using Wg expression as a marker of Notch activation, we identified exceptions to this (Fig. 3b, f), and these exceptional clones respect the compartment border (3 out of 3 *Notch*^{55e11} and 35 out of 35 *Dl Ser* mutant clones with intact Wg respected the border, whereas 11 out of 11 *Notch*^{55e11} and 47 out of 49 *Dl Ser* mutant clones with disrupted Wg did not). The correlation between loss of Notch signalling and violation of the compartment border implies that it is activation of Notch that is essential.

Unlike Notch or *Dl Ser* is normally expressed specifically by

Table 2 Influence of ectopic Fng expression on compartmental location

Flip-out Fng ⁺ clones				Flip-out control clones			
Identity	No.	Location	No.	Identity	No.	Location	No.
0–48 h							
Dorsal	24	D—internal	19	Dorsal	29	D—internal	17
		D—border	5			D—border	12
		Ventral	0			Ventral	0
Ventral	8	V—internal	3	Ventral	13	V—internal	8
		V—border	0			V—border	5
		Dorsal	5			Dorsal	0
Mixed*	26	Dorsal	26	Mixed*	50	Dorsal	0
		Ventral	0			Ventral	50
48–72 h							
Dorsal	83	D—internal	62	Dorsal	94	D—internal	65
		D—border	21			D—border	29
		Ventral	0			Ventral	0
Ventral	64	V—internal	50	Ventral	73	V—internal	42
		V—border	0			V—border	31
		Dorsal	14			Dorsal	0
Mixed*	4	Dorsal	4	Mixed*	9	Dorsal	0
		Ventral	0			Ventral	9

Clones were induced at the times shown. Dorsal or ventral identity was defined by *ap-lacZ* expression. Locations were scored as dorsal if any part of the clone appeared on the dorsal side of the D–V border.

* Among clones with mixed identity, only non-*ap*-expressing cells were scored.

dorsal cells. We therefore analysed Flip-out Ser clones to assess the influence of Ser on compartmentalization further. Activation of Notch by Ser in dorsal cells is prevented by Fng^{10,11}. In addition, high-level expression of Notch ligands can result in autonomous inhibition of Notch signalling²³. The influence of ectopic Ser expression on Notch activation in our Flip-out clones was variable; in some cases Notch was activated throughout ventral Ser-expressing clones, and in some cases Notch signalling was inhibited within these clones. Ventral or mixed identity Flip-out Ser clones that touch the D–V boundary and inhibit and endogenous Notch activation always (10 out of 10) disturbed D–V compartmentalization (Fig. 4b), whereas most (12 out of 18) clones that appeared to leave endogenous Notch activation intact did not disturb compartmentalization (Fig. 4a).

A consistent implication of the above experiments is that activation of Notch along the interface between dorsal and ventral cells is essential for their separation into distinct compartments. This also explains violations of the compartment border induced by *fng*. Clones of dorsal cells that are mutant for *fng*, and clones of ventral cells that ectopically express Fng, disrupt Notch activation when they contact the normal D–V interface. In addition, *fng* mutant clones, Flip-out Fng clones and Flip-out Ser clones can also induce an ectopic stripe of Notch activation. The relative straightness of this stripe (Figs 1, 2 and 4), as well as its incorporation into the stripe of Notch activation in flanking cells, implies that a stripe of Notch activation is actually the essential feature of D–V compartmentalization.

However, Notch signalling does not specify distinct dorsal or ventral cell affinities. Clones of cells that are mutant for *Notch* or its ligands can violate the compartment border from either side (Fig. 3). Moreover, clones of cells that are mutant for *Notch* or its ligands can also induce non-autonomous violations of the compartment boundary by wild-type cells (Fig. 3b, c, h). The impression, therefore, is that a stripe of Notch activation creates a fence that separates dorsal and ventral cells; when the fence is broken, cells can intermix in either direction.

To assess this further, we examined Flip-out clones that express the intracellular domain of Notch, which is constitutively activated for signalling^{16,24}. As judged by *ap-lacZ* staining, these clones can

respect the D–V compartment border from either the dorsal (16 out of 16) or the ventral (16 out of 16) side (Fig. 4c), and clones that are produced early enough to be of mixed dorsal and ventral identity can extend across the D–V border without disrupting compartmentalization (14 out of 16) (Fig. 4d).

As the edge of *ap* expression is straight within a broader region of Notch activation (Fig. 4d), there must exist additional mechanisms that contribute to compartmentalization. To examine whether Fng participates in these additional mechanisms, we analysed Flip-out clones co-expressing Fng and activated Notch. If the only role of Fng in D–V compartmentalization were to position Notch activation, then ubiquitous Notch activation should fully suppress the effects of Fng. However, ventral cells co-expressing Fng and activated Notch exhibited a slight but consistent (29 out of 36 clones) extension into the dorsal compartment (Fig. 4e, f). As Wg expression suggests that our activated-Notch clones have levels of Notch signalling similar to that at the normal D–V boundary (Fig. 4c, d), this result suggests that Fng may have an effect on D–V compartmentalization that is separate from its influence on Notch activation.

Studies in the *Drosophila* wing and abdomen have revealed that signalling from posterior to anterior cells has an essential role in effecting anterior–posterior compartmentalization, but that additional factors also contribute to this process^{25–27}. Similarly, our results imply that multiple mechanisms contribute to D–V compartmentalization. The primary mechanism is establishment of a stripe of Notch activation along the interface between dorsal and ventral cells. Fng positions this stripe of Notch activation, but it also seems to participate in a secondary mechanism that can influence cell behaviours within a larger region of Notch activation. In the wing, this secondary mechanism may help to ensure the precise register of the compartment border with *ap* expression²⁸. Fng-dependent separation of adjacent cell populations will probably also be important for the development of other tissues in which *Fng*-related genes are involved in making developmental boundaries²⁹. □

Methods

*fng*¹³, *D^{fr}10* *Ser*^{RX106}, *Notch*^{55e11} or *Notch*^{CO} mutant clones were generated 52–72 h after egg laying by Flipase-mediated mitotic recombination¹⁸. Ectopic expression of Fng, activated Notch or Ser was performed using the UAS-Gal4 and Flip-out systems to make clones of cells expressing Gal4 in animals with *UAS-fng27*, *UAS-Ser* or *UAS-ΔN34a* transgenes¹⁸. Immunostaining was performed as described previously^{10,18}, with the addition of *ap* detection using the *ap-lacZ* enhancer trap line *rk568* (ref. 6), and immunostaining of *Notch*^{55e11} or *Notch*^{CO} clones with the Notch monoclonal C458.2H.

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Silencing of TGF- β signalling by the pseudoreceptor BAMBI

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Members of the transforming growth factor- β (TGF- β) superfamily, including TGF- β , bone morphogenetic proteins (BMPs), activins and nodals, are vital for regulating growth and differentiation¹. These growth factors transduce their signals through pairs of transmembrane type I and type II receptor kinases^{2–4}. Here, we have cloned a transmembrane protein, BAMBI, which is related to TGF- β -family type I receptors but lacks an intracellular kinase domain. We show that BAMBI is co-

expressed with the ventralizing morphogen BMP4 (refs 5, 6) during *Xenopus* embryogenesis and that it requires BMP signalling for its expression. The protein stably associates with TGF- β -family receptors and inhibits BMP and activin as well as TGF- β signalling. Finally, we provide evidence that BAMBI's inhibitory effects are mediated by its intracellular domain, which resembles the homodimerization interface of a type I receptor and prevents the formation of receptor complexes. The results indicate that BAMBI negatively regulates TGF- β -family signalling by a regulatory mechanism involving the interaction of signalling receptors with a pseudoreceptor.

In an expression screen for components involved in BMP4 signalling⁷, we identified a novel *Xenopus* complementary DNA whose expression pattern is very similar to that of BMP4 (Fig. 1b). The predicted gene product, named BAMBI (BMP and activin membrane-bound inhibitor), shows 89% amino-acid-sequence similarity and 83% identity to *nma*, a human gene without known function which is downregulated in metastatic melanoma cell lines⁸. BAMBI encodes a putative transmembrane protein of 260 amino acids with an extracellular domain that is closely related to those of type I TGF- β receptors, and among these shows most similarity (53%) to *Xenopus* BMP receptor type-I (BMPRI; Fig. 1a)^{9,10}. Unlike all other known TGF- β type I or type II receptors, the intracellular domain of BAMBI is relatively short and does not

encode a serine/threonine-kinase domain, although it shares some sequence homology with the E6 and catalytic loops of receptor serine/threonine kinases (see Supplementary Information). The results indicate that BAMBI encodes a novel TGF- β receptor-related protein.

Embryonic expression of BAMBI closely follows that of *Xenopus* BMP4 (Fig. 1b)^{11,12}. Using polymerase chain reaction with reverse transcription (RT-PCR) we can detect a maternal BAMBI transcript before the onset of zygotic transcription (stage 9), but the highest expression is observed during gastrulation and neurulation (Fig. 1c). RT-PCR analysis shows that BAMBI is induced in dorsal marginal zone (DMZ) explants from *Xenopus* embryos microinjected with BMP4 messenger RNA (Fig. 1d). BMP signalling is also required for BAMBI, as its expression is downregulated in ventral marginal zone (VMZ) explants from embryos microinjected with a dominant-negative BMP2/4 receptor that prevents BMP signalling¹⁰ (Fig. 1d). We conclude that BAMBI is co-expressed with and regulated by BMP4.

These properties indicated that BAMBI might affect BMP signalling in *Xenopus* embryos. RT-PCR analysis (Fig. 2a) shows that microinjection of BAMBI mRNA interferes in animal cap assays with *Xbra* induction by BMP4 or by activin, but not by FGF. To corroborate these findings we carried out phenotypic rescue assays. Activin can induce mesoderm in animal caps, which leads to a

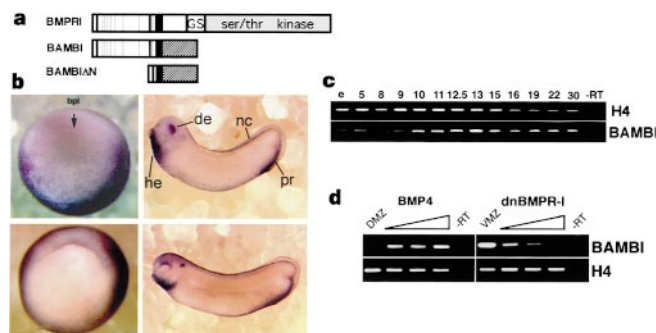


Figure 1 BAMBI is related to BMPRI and is regulated by BMP4. **a**, Drawing of BMPRI and BAMBI. Left to right: signal sequence (grey box); putative extracellular domains (white box) containing seven conserved cysteines (vertical lines) and cysteine box (thick vertical line); transmembrane domain (black box); GS and intracellular Ser/Thr-kinase domains of BMPRI; and intracellular domain of BAMBI (hatched). In BAMBI Δ N the extracellular domain is deleted. **b**, Co-expression of BAMBI (top) and BMP4 (bottom) in *Xenopus* gastrulae (stage 10; left) and tadpoles (right). Embryos are shown dorsal side up in vegetal view (gastrulae) or lateral view (tadpoles). bpl, dorsal blastopore lip; de, dorsal eye; he, heart; nc, neural crest; pr, proctodeum. **c**, RT-PCR analysis of BAMBI expression in embryos of the indicated stages. –RT, minus reverse transcription control sample; H4, histone H4 for normalization. **d**, DMZ and VMZ assays: 4-cell embryos were uninjected or microinjected into each blastomere with 0.025, 0.1 or 0.3 ng BMP4, or 0.1, 0.25 or 0.5 ng dominant-negative BMP2/4 receptor (dnBMPRI) mRNA, as indicated. Dorsal (DMZ) or ventral marginal zone (VMZ) fragments were cut from early gastrulae and analysed for induction of BAMBI at stage 10.5 by RT-PCR.

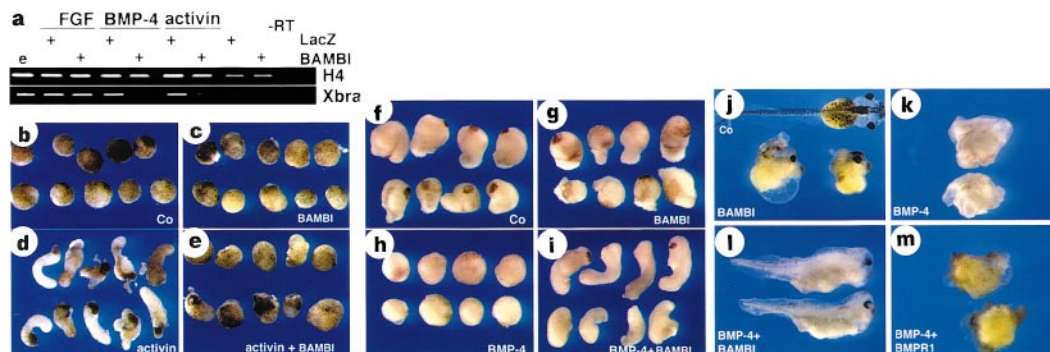


Figure 2 BAMBI inhibits BMP and activin signalling in *Xenopus* embryos. **a**, 4-cell embryos were microinjected into all blastomeres with 0.75 ng per blastomere control (lacZ) or BAMBI mRNA. Animal caps were excised from blastula embryos and cultivated with 1 μ g ml⁻¹ bFGF, 1 μ g ml⁻¹ BMP4 or 20 ng ml⁻¹ activin as indicated, until stage 10. Expression of histone 4 (H4, for normalization) and the mesodermal marker *Xbra* were analysed by RT-PCR. **b–e**, Animal cap assays. 4-cell embryos were microinjected into all blastomeres with 0.5 ng per blastomere control (lacZ) or BAMBI mRNA with or without 0.1 ng activin mRNA as indicated. Animal caps were excised from blastula embryos and cultivated until stage 20 to score elongation of explants, indicative of mesoderm induction. **f–i**, DMZ assays. 4-cell embryos were microinjected into each blastomere with 0.5 ng

control (lacZ) or 0.4 ng BAMBI, with or without 0.4 ng BMP4 mRNA per blastomere as indicated. DMZs were excised from gastrula embryos and cultivated until stage 20 to score cement-gland formation (brown tissue) and elongation of explants, indicative of dorsal mesoderm differentiation. **j**, 4-cell embryos were uninjected (top) or microinjected into each blastomere with 0.8 ng BAMBI mRNA, leading to gastrulation defects, spina bifida and enlarged heads (63%; $n = 153$). **k–m**, Rescue of BMP4-injected embryos. 4-cell embryos were microinjected into each blastomere with 0.5 ng BMP4, with or without 0.5 ng BAMBI or BMPRI mRNA. Note loss of head and axial structures following BMP4 injection, which is rescued from a dorsanterior index (DAI) of 1.7 ($n = 68$) to 3.1 ($n = 72$) by co-injection of BAMBI but not BMPRI (DAI = 1.5; $n = 71$).

characteristic elongation of the explants (Fig. 2b, d). Co-expression of activin and BAMBI prevents this elongation, indicating inhibition of activin signalling (Fig. 2c, e). In a similar assay, DMZ explants will autonomously elongate and differentiate dark cement-gland tissue, and this can be inhibited by microinjection of BMP4 mRNA (Fig. 2f, h). Co-injection of BMP4 with BAMBI mRNA reverts this phenotype, leading to elongation and cement-gland differentiation (Fig. 2g, i).

As BAMBI can interfere with activin and BMP4 signalling in explant assays, we tested its effects on embryonic development. Embryos microinjected with BAMBI mRNA show a weak dorso-anteriorized phenotype with enlarged heads, and at higher doses they suffer from gastrulation defects resulting in truncated trunk/tail structures (Fig. 2j). The dorso-anteriorized phenotype is consistent with BAMBI's inhibiting BMP signalling, which normally antagonizes dorso-anterior structures^{3,6}. Consistent with this, co-injection of BAMBI, but not BMPR-I, mRNA rescues BMP4-ventralized embryos, resulting in fairly normal tadpoles (Fig. 2k–m). In conclusion, the effects observed following overexpression in embryonic explants and whole embryos indicate that BAMBI antagonizes BMP and activin signalling.

To test whether BAMBI has any effect on BMP signalling through endogenous receptors in mammalian cells, the BMP-responsive reporter pVent-Luc¹³ was transfected into mouse embryonic carcinoma P19 cells with or without BAMBI. BMP2 induced expression of this reporter in P19 cells, as previously shown¹⁴, and BAMBI significantly inhibited this effect (Fig. 3a). A construct BAMBIΔN with its extracellular domain deleted did not affect endogenous BMP signalling but reversed the inhibitory effects of wild-type BAMBI (Fig. 3a), by acting as a dominant-negative variant (see below). In similar experiments using the activin/TGF-β-responsive Mix.2 ARE reporter pA3-Luc¹⁵, BAMBI blocked the activation of this reporter elicited by activin through endogenous receptors in P19 cells (Fig. 3b).

To extend these observations to other type I receptors, and to distinguish between a ligand-dependent and a ligand-independent mode of action for BAMBI, we tested the effect of BAMBI on signalling by constitutively active forms of the type I receptors. We tested the BMP2/4 type I receptors BMPR-IA and BMPR-IB (also known as ALK3 and ALK6, respectively), the orphan receptor

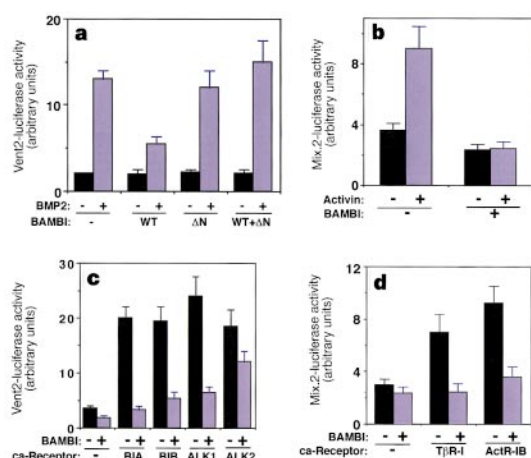


Figure 3 BAMBI inhibits ligand-independent signalling by TGF-β family receptors. The pVent2-Luc reporter (a, c) or the pA3-Luc reporter (b, d) were transiently transfected into P19 cells together, with or without BAMBI, BAMBIΔN (ΔN) or constitutively active type I receptor constructs (ca-receptor), as indicated. After 24 h, cells received 0.5 nM BMP2 (a), 2 nM activin (b) or no additions. Luciferase activity was determined 20 h later. Otherwise, luciferase activity was measured 2 days after transfection (b, d). Data are the average of three or more assays ± s.d.. BIA, BIB are constitutively active BMPR-IA and BMPR-IB, respectively.

ALK1, the putative BMP7 receptor ALK2, the TGF-β type I receptor TβR-I (also known as ALK5) and the activin type I receptor ActR-IB (also known as ALK4) (Fig. 3c, d). Transfection of constitutively active BMPR-IA, BMPR-IB, ALK1 or ALK2 into P19 cells activated the pVent-Luc reporter¹⁶ (Fig. 3c), whereas transfection of constitutively active TβR-I or ActR-IB activated the pA3-Luc reporter (Fig. 3d). Co-expression of BAMBI decreased the activation of these reporter genes by the constitutively active type I receptors (Fig. 3c, d). The ability of BAMBI to inhibit signalling by constitutively active type I receptors indicates that BAMBI may not need to interfere with ligand binding in order to inhibit receptor signalling.

We used co-immunoprecipitation to investigate whether BAMBI can interact with TGF-β receptors. We found an interaction between BAMBI and all of the type I receptors except ALK2, as

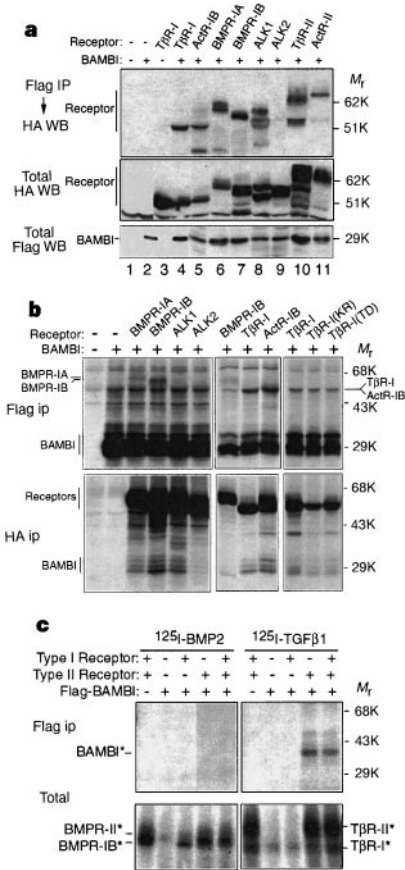


Figure 4 BAMBI interacts with TGF-β receptors. **a**, BAMBI binds to TGF-β receptors. To reveal receptor–BAMBI complexes, the lysates from COS1 cells transfected with the C-terminally HA-tagged type I receptors and C-terminally Flag-tagged BAMBI were immunoprecipitated with anti-Flag M2 antibody and analysed by western blotting with anti-HA polyclonal antibody (top). The expression of receptors and BAMBI was checked by western blotting of total cell lysate (5% aliquots) with anti-HA and anti-Flag antibodies, respectively (middle and bottom). **b**, Cells transfected with various constructs as indicated were labelled with [³⁵S]Met/Cys for 3 h and lysed. Two-thirds of the lysate were subjected to anti-Flag immunoprecipitation and one-third was subjected to anti-HA immunoprecipitation. Proteins were visualized by SDS-PAGE and fluorography for 16 h (middle and right) or 72 h (left). **c**, Transfected cells were incubated with ¹²⁵I-BMP2 or ¹²⁵I-TGF-β1 and the crosslinking agent DSS. Affinity-labelled cell lysates were subjected to anti-Flag immunoprecipitation and visualized by SDS-PAGE and autoradiography. The lower panels show receptor labelling by analysing aliquots of total cell lysate. Names with asterisks indicate the positions corresponding to BAMBI–ligand and receptor–ligand crosslinking products (ligand monomer *M_r* = 13K). BAMBI levels were similar in all conditions, as determined by anti-Flag immunoblotting (not shown).

well as an interaction with the type II receptors T β R-II and ActR-II (Fig. 4a). Anti-Flag immunoprecipitations from the metabolically labelled transfectants (Fig. 4b) showed that Flag-BAMBI associates with T β R-I, ActR-IB, BMPR-IB and BMPR-IA (in order of affinity). Anti-HA immunoprecipitations revealed that BAMBI associates with T β R-I, ActR-IB, BMPR-IB, ALK1 and BMPR-IA (in order of

affinity), and does not interact with ALK2. BAMBI also interacts with the constitutively active T β R-I mutant T β R-I(TD) and with the kinase-inactive mutant T β R-I(KR) (Fig. 4b).

BAMBI alone did not bind [125 I]BMP2 or [125 I]TGF- β 1, as determined using a receptor affinity-labelling protocol¹⁷. However, BAMBI contacted TGF- β 1 when co-expressed with the TGF- β type

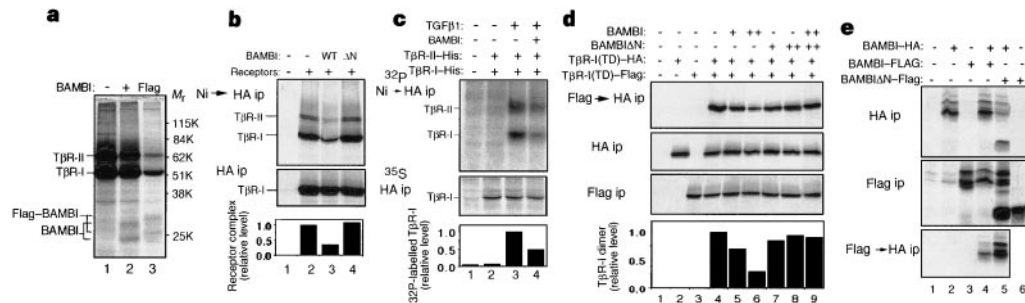


Figure 5 BAMBI inhibits formation of functional receptor complex. **a**, BAMBI is incorporated into a receptor complex. Cells transfected with T β R-II and T β R-I, with or without BAMBI, were treated with TGF- β and labelled with [35 S]Met/Cys. Then receptor complexes were isolated by sequential precipitation using a hexahistidine tag in the T β R-II construct (T β R-II-his) and an HA-tag in the T β R-I construct (T β R-I-HA) (lanes 1 and 2), or using a hexahistidine tag in T β R-II and a Flag tag in the BAMBI construct (lane 3). **b**, **c**, BAMBI inhibits receptor-complex formation. Cells transfected with T β R-II-his and T β R-I-HA with various BAMBI, as indicated, were treated with TGF β and metabolically labelled with [35 S]Met/Cys (**b**) or [32 P]orthophosphate (**c**). Receptor complexes were purified as in **a**. T β R-I expression was confirmed by anti-HA immunoprecipitation.

Histograms show levels of receptor complexes or 32 P-labelled T β R-I. **d**, BAMBI inhibits T β R-I(TD) homocomplex formation. Cells transfected with various constructs were labelled with [35 S]Met/Cys and T β R-I(TD) homocomplex was purified by sequential immunoprecipitation with anti-Flag and then with anti-HA antibodies. Receptor expression was confirmed by anti-HA or anti-Flag immunoprecipitation. Histograms show quantification of homocomplex level. **e**, BAMBI forms a homocomplex. Cells transfected with various constructs were labelled with [35 S]Met/Cys and BAMBI homocomplex was purified by sequential immunoprecipitation with anti-Flag and then with anti-HA antibodies. BAMBI expression was confirmed by anti-HA or anti-Flag immunoprecipitation.

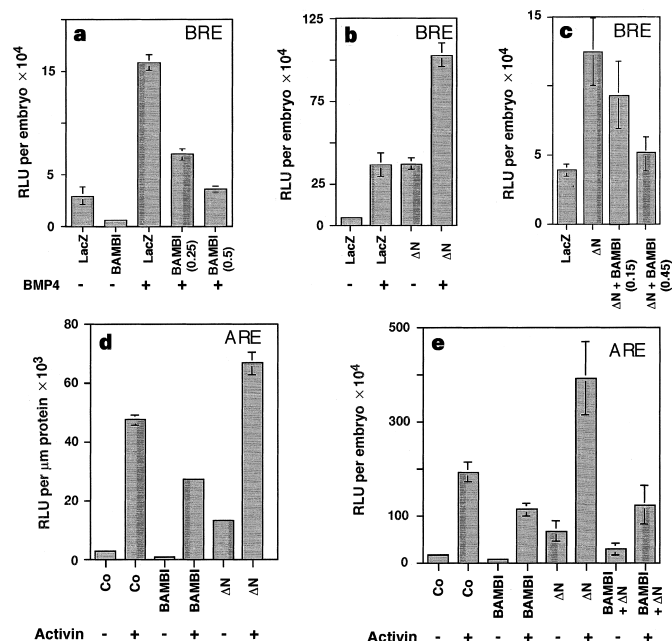


Figure 6 The intracellular domain of BAMBI mediates inhibition of TGF- β family signalling in *Xenopus*. **a–c**, 4-cell embryos were microinjected into each blastomere with 25 pg of the BMP-responsive reporter plasmid pVent2-Luc (BRE) and the following mRNAs (in ng per blastomere) as indicated: 1.0 control (lacZ), 0.5 BMP4, 0.5 BAMBI (unless indicated otherwise in parenthesis), 0.5 BAMBI Δ N. At stage 10, luciferase assays were carried out with extracts of whole embryos at least in triplicate. RLU, relative light units. **d**, **e**, 4-cell embryos were microinjected anally (**d**) or equatorially (**e**) into each blastomere with 25 pg of the activin-responsive reporter plasmid pXFD1'Luc-i (ARE) and the following mRNAs in ng per blastomere as indicated: 0.5 control (lacZ or PPL), 0.15 activin, 0.5 BAMBI, 0.25 BAMBI Δ N. At gastrula stage, animal caps were excised (**d**) or whole embryos were sampled (**e**) and luciferase and total protein assays carried out. Luciferase activity in RLU is normalized to protein (**d**) or to embryos (**e**). **f**, Model for silencing by BAMBI. Top: following ligand binding, type II receptors associate with preassembled type I receptor homodimers, leading to receptor transphosphorylation and TGF- β signal transduction. Bottom: its extracellular domain allows BAMBI to dock to the receptor complex while the intracellular domain interferes with homodimeric type I receptor interactions^{19,20,29,30}, by intercalating between and/or outcompeting receptor subunits.

II receptor (T β R-II) (Fig. 4c, top). As T β R-II is a primary ligand-binding component in the TGF- β receptor complex¹⁸, this result indicates that BAMBI may be incorporated into complexes with T β R-II. We confirmed this by analysing the association of BAMBI with cotransfected T β R-I and T β R-II in the presence of ligand. Using a protocol to isolate the heterotetrameric T β R-I/T β R-II receptor complex¹⁸, we found that BAMBI is coprecipitated with this complex (Fig. 5a, lane 2). Conversely, T β R-I and T β R-II can be co-immunoprecipitated with Flag-tagged BAMBI (Fig. 5a, lane 3).

Expression of BAMBI caused a decrease in T β R-I/T β R-II complex (Fig. 5b) and in the amount of phosphorylated T β R-I in this complex (Fig. 5c). These effects may underlie the ability of BAMBI to inhibit TGF- β signalling, although interference with type II receptors is not excluded. BAMBI pre-assembled with T β R-I may decrease the level of T β R-I/T β R-II complex by taking the place of one T β R-I molecule or decreasing the stability of this complex. Furthermore, the ability of BAMBI to interact with type I TGF- β receptors may also explain its ability to inhibit signalling by constitutively active forms of these receptors: BAMBI inhibits the dimerization of T β R-I(TD) (Fig. 5d). Dimerization is required for signalling by T β R-I(TD)^{19,20}.

To study the intracellular domain of BAMBI further, we analysed BAMBI Δ N (Fig. 1a). BAMBI Δ N had no significant effect on the BMP2 response in P19 cells, but neutralized the inhibitory effect of wild-type BAMBI in these cells (Fig. 3a). BAMBI dimerizes with itself and also with BAMBI Δ N (Fig. 5e), which indicates that BAMBI Δ N may work by sequestering wild-type BAMBI. Indeed, BAMBI Δ N does not interact with T β R-I (data not shown), but prevents BAMBI from inhibiting the dimerization of T β R-I(TD) (Fig. 5d, lane 9). Collectively, these results indicate that BAMBI Δ N may act as a dominant-negative BAMBI inhibitor.

We used the dominant-negative BAMBI Δ N to assess the role of endogenous BAMBI in *Xenopus* embryos. Figure 6a shows that basal luciferase activity due to endogenous BMP signalling and BMP4-stimulated luciferase activity are both inhibited by BAMBI. In contrast to the wild type, BAMBI Δ N strongly activates endogenous BMP signalling and BMP4-induced luciferase activity (Fig. 6b). Similarly, wild-type BAMBI inhibits endogenous and activin-induced luciferase activity in animal caps and whole embryos (Fig. 6d, e). In contrast, BAMBI Δ N activates endogenous and activin-induced luciferase activity. The activating effect of BAMBI Δ N on BMP and activin signalling is specific, as it can be reversed by co-expression with the wild-type mRNA in both cases (Fig. 6c, e). The results indicate that both BMP and activin-like signalling are subject to negative, BAMBI-dependent regulation in *Xenopus* embryos.

Figure 6f shows a model of BAMBI function. Signalling by BAMBI may be relevant for limiting the signalling range of BMPs and activins, which are thought to act as morphogens during early embryogenesis⁵. □

Methods

In vitro fertilization, embryo culture, staging, microinjection, culture of explants and whole-mount *in situ* hybridization were carried out as described²¹.

Biochemical assays

Epitope-tag antibodies were from Santa Cruz Biotechnology. Metabolic labelling, receptor-affinity labelling, immunoprecipitation and receptor-complex isolation were performed as described²². The cotransfected BAMBI and receptors were expressed at relative levels ranging from 1:1 to 1:3, depending on the receptor. To isolate T β R-I or BAMBI complexes, ³⁵S-labelled cells were lysed with lysis buffer (10 mM Tris, pH 7.8; 150 mM NaCl; 0.5% NP-40) and protease inhibitors. Cell lysates were incubated with anti-Flag M2 antibody (Sigma) and protein A Sepharose for 4 h and the immunoprecipitates were washed with lysis buffer. After boiling in a 1% SDS buffer, eluates were diluted to 0.1% SDS and immunoprecipitated with anti-HA 12CA5 antibody.

Constructs

A full-length BAMBI clone (pBAMBI, in pBSII-KS) was isolated by expression-screening a neurula-stage plasmid library by *in situ* whole-mount hybridization⁷ (EMBL accession

number AJ243576). BAMBI, BAMBI Δ N (lacking amino acids 30–132) and carboxy-terminally FLAG-epitope-tagged BAMBI were subcloned into pCS2+²³ and a C-terminal HA-epitope-tagged form was subcloned into pCMV5. The receptor DNA constructs have been described¹⁸.

Gene expression assays

RT-PCR assays were carried out in the exponential phase of amplification as described²¹. BAMBI primers were (fw)GAGCGACTCG GAGGGATTACAA (r)GTACCCAGCGT CACCATCCAGA (154 base pairs (bp)). Luciferase assays were carried out as described with P19 cells¹⁶ and *Xenopus*²⁴. The luciferase reporter constructs employed were the pARE-Luc from the Mix2 promoter²⁵, pXvent-Luc (BRE)²⁶ and pXFD1'Luc-i (ARE) encompassing the 1.6-kilobase (kb) intron of the XFD1' gene, which contains an ARE that is directly activated by Smad2 (ref. 27), fused to the XFD1 minimal promoter (bp –80 to +21 of the transcription start site²⁸; a gift from W. Knöchel).

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Tom22 is a multifunctional organizer of the mitochondrial preprotein translocase

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Mitochondrial preproteins are imported by a multisubunit translocase of the outer membrane (TOM), including receptor proteins and a general import pore^{1–5}. The central receptor Tom22 binds preproteins through both its cytosolic domain and its intermembrane space domain^{6–10} and is stably associated with the channel protein Tom40 (refs 11–13). Here we report the unexpected observation that a yeast strain can survive without Tom22, although it is strongly reduced in growth and the import of mitochondrial proteins. Tom22 is a multifunctional protein that is required for the higher-level organization of the TOM machinery. In the absence of Tom22, the translocase dissociates into core complexes, representing the basic import units, but lacks a tight control of channel gating. The single membrane anchor of Tom22 is required for a stable interaction between the core complexes, whereas its cytosolic domain serves as docking point for the peripheral receptors Tom20 and Tom70. Thus a preprotein translocase can combine receptor functions with distinct organizing roles in a multidomain protein.

Only two Tom proteins, Tom22 and Tom40, have been shown to be essential for cell viability^{6,14,15}, as determined by a standard assay, namely disruption of one chromosomal copy of a gene in a diploid strain of the yeast *Saccharomyces cerevisiae*, followed by sporulation and analysis to determine whether the resulting haploid cells lacking the gene can grow¹⁶. Similarly, a sheltered disruption of *TOM22* in *Neurospora crassa* indicated that Tom22 is essential for cell viability¹⁷. In a second approach using *S. cerevisiae*, we investigated whether *TOM22* could be deleted directly from haploid cells by a one-step disruption, but no viable cells lacking *TOM22* were obtained. In a third approach, a diploid yeast strain (OL551) lacking the *TOM22* coding region in one of its chromosomal copies received a plasmid containing *TOM22* and the *URA3* marker. After sporulation, haploid cells containing the chromosomal disruption of *TOM22* were selected, all of which carried the *TOM22*–*URA3*

plasmid. By adding 5-fluoroorotic acid (5-FOA), we found that cells (OL201) were selected that had lost the *URA3*–*TOM22* plasmid and seemed to lack *TOM22*.

To exclude the possibility that only the *URA3* marker was inactivated while the *TOM22* gene was still present in OL201, we performed analytical polymerase chain reaction (PCR) with primers flanking the *TOM22* gene. OL201 contained only the disrupted gene (Fig. 1a, lane 3), the control haploid strain OL223 contained the wild-type *TOM22* gene (Fig. 1a, lane 2), and the diploid strain contained both the wild-type gene and the disrupted gene (Fig. 1a, lane 1). The absence of the *TOM22* coding region in OL201 was confirmed by Southern blot analysis, and northern blot analysis revealed the complete lack of *TOM22* messenger RNA (not shown). We conclude that the OL201 strain lacks the *TOM22* gene, and so we now refer to it as *tom22Δ*. The *tom22Δ* cells were devoid of mitochondrial DNA (*rho*⁰). The control haploid strain (OL223) was converted to the *rho*⁰ state (referred to as wild type with regard to *TOM22*). On fermentable medium, the growth of *tom22Δ* cells was about fourfold slower than that of OL223 cells.

The frequency of spontaneous loss of the *URA3* plasmid from a control strain was about 0.1, whereas the frequency for obtaining OL201 was ~0.02. This indicates that around 20% of the cells could survive the loss of *TOM22*. Moreover, a backcross of OL201 with the wild-type haploid also failed to provide evidence for a rapid generation of extragenic suppressor mutations to explain the viability of *tom22Δ* cells. The analysis of more than 90 tetrads revealed a 2:2 segregation of viability, and all viable spores contained the wild-type *TOM22* gene, indicating that *tom22Δ* spores cannot germinate productively. The strong growth defects of *tom22Δ* cells provide a likely explanation of why the cells did not survive two standard procedures for gene deletion, leading to the previous assumption that Tom22 is essential for viability. Only the mild approach involving plasmid loss allowed viable *tom22Δ* cells to be recovered.

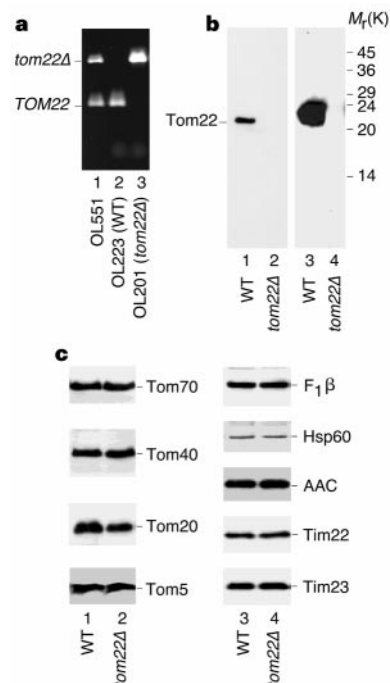


Figure 1 A yeast strain lacking *TOM22*. **a**, Analytical PCR yielding fragments typical for the intact *TOM22* gene (409 base pairs (bp); WT, wild type) and the *HIS3*-disrupted *TOM22* gene (1,110 bp; *tom22Δ*). **b**, **c**, Analysis of the protein composition of *tom22Δ* mitochondria. Isolated mitochondria (25 μg of protein) were separated by SDS-PAGE and analysed by immunodecoration. In **b**, lanes 3 and 4 received 200 μg of mitochondrial protein.

Mitochondria were isolated from *tom22Δ* cells and the corresponding wild type. Immunodecoration demonstrated that the *tom22Δ* mitochondria completely lacked Tom22 (Fig. 1b, lane 2). No smaller fragments reacting with a polyclonal anti-Tom22 serum were detected, even after extensive loading of the gel (Fig. 1b, lane 4). The amount of Tom20 in *tom22Δ* mitochondria was reduced to about 65% of that in the wild type (Fig. 1c, lane 2). All other proteins tested were found in similar amounts in wild-type and mutant mitochondria, including Tom70, Tom40 and Tom5 of the outer membrane (Fig. 1c, lanes 1 and 2), Tim23, Tim22 and the ADP/ATP carrier (AAC) of the inner membrane, the β -subunit of the F_1F_0 -ATPase ($F_1\beta$) and the matrix chaperonin Hsp60 (Fig. 1c, lanes 3 and 4).

To assay the protein import capacity of *tom22Δ* mitochondria, *in vitro*-synthesized 35 S-labelled preproteins were incubated with the isolated mitochondria (Fig. 2a–c). Preproteins containing cleavable presequences were used that were destined for the intermembrane space (cytochrome *b*₅; not shown), the matrix side of the inner membrane ($F_1\beta$) or the matrix (the α -subunit of the matrix processing peptidase and a fusion protein between the presequence of F_0F_1 -ATPase subunit 9 and dihydrofolate reductase). Non-cleavable precursors of AAC and the outer-membrane protein porin were also used. We found that *tom22Δ* mitochondria were able to import preproteins, but with a strongly reduced efficiency of around 5–15% compared with the wild-type mitochondria (Fig. 2a, b, compare lanes 5–7 with 1–3; Fig. 2c, compare lanes 7–11 with 1–

5). The import into or across the inner membrane of *tom22Δ* mitochondria was blocked by dissipation of the membrane potential (Fig. 2a, b, lanes 8; Fig. 2c, lane 12). By re-expression of Tom22 from a plasmid carrying the *TOM22* gene, protein import was largely restored (Fig. 2b, lanes 9–11), demonstrating that the strong effects on protein import were specific to the loss of Tom22 (reduction of Tom20 content would only slightly inhibit the import of cleavable preproteins to around 80% of that of the wild-type¹⁸).

To monitor the association of preproteins with Tom proteins, we used an AAC–DHFR fusion protein. Addition of methotrexate stabilizes the carboxy-terminal DHFR so it does not cross the outer membrane¹⁹, leading to the arrest of AAC–DHFR in the TOM machinery. When the cross-linking reagent ethylene glycol bis-(succinimidylsuccinate) (EGS) was added, Tom proteins in the vicinity of the preprotein were identified by immunoprecipitation under stringent conditions. The efficiency of crosslinking of AAC–DHFR to the first receptor Tom70 was similar in wild-type and *tom22Δ* mitochondria (Fig. 2d, lanes 1 and 6), indicating that the lack of Tom22 did not impair the initial binding of the preprotein to the mitochondrial surface. However, crosslinking of AAC–DHFR to Tom40 was strongly reduced in *tom22Δ* mitochondria (Fig. 2d, lane 7), indicating that transfer of the preprotein into the import pore was inhibited. Crosslinking to the second receptor Tom20 was also reduced (Fig. 2d, lane 8), indicating that the lack of Tom22 may impair the transfer of preprotein from Tom70 to Tom20. Antibodies

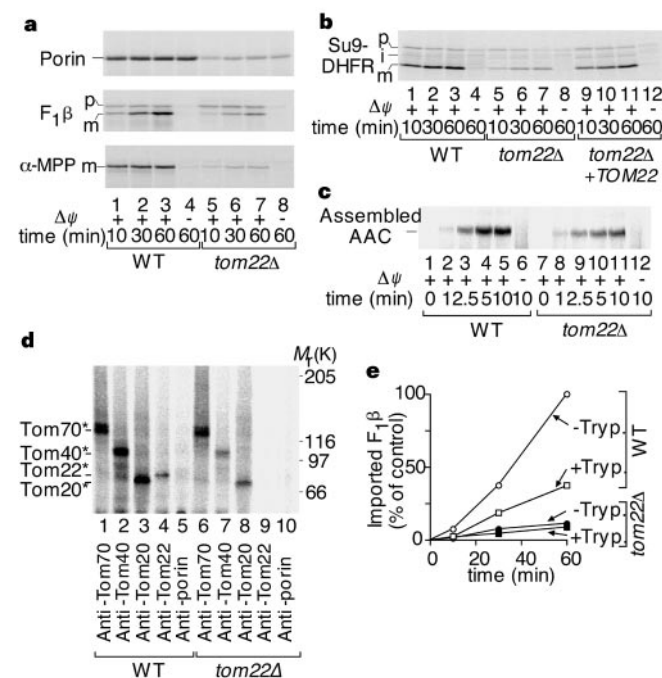


Figure 2 Inhibition of preprotein import into *tom22Δ* mitochondria. **a**, 35 S-labelled preproteins were imported into isolated wild-type or *tom22Δ* mitochondria in the presence or absence of a membrane potential ($\Delta\psi$). After treatment with proteinase K, the reisolated mitochondria were subjected to SDS-PAGE and storage phosphorimaging technology. **b**, Restoration of protein import by re-expressing Tom22, including mitochondria isolated from a *tom22Δ* strain (lanes 9–12). Restoration of protein import was comparable if Tom22 was re-expressed from either a multicopy vector (Yep13) or a single-copy vector (pRS415). p, i, m indicate precursor-, intermediate- and mature-sized forms of a protein, respectively. **c**, Import and assembly of AAC are inhibited. Assembly to the dimeric form was assessed by blue native PAGE. **d**, Crosslinking of AAC. AAC–DHFR was accumulated across the outer membrane in the presence of methotrexate. Reisolated mitochondria were crosslinked with EGS and analysed by immunoprecipitation. **e**, Bypass import into *tom22Δ* mitochondria. Where indicated, mitochondria were pretreated with trypsin (tryp.) before the import of $F_1\beta$. Protein import into non-trypsinized wild-type mitochondria after 60 min was set to 100% (control).

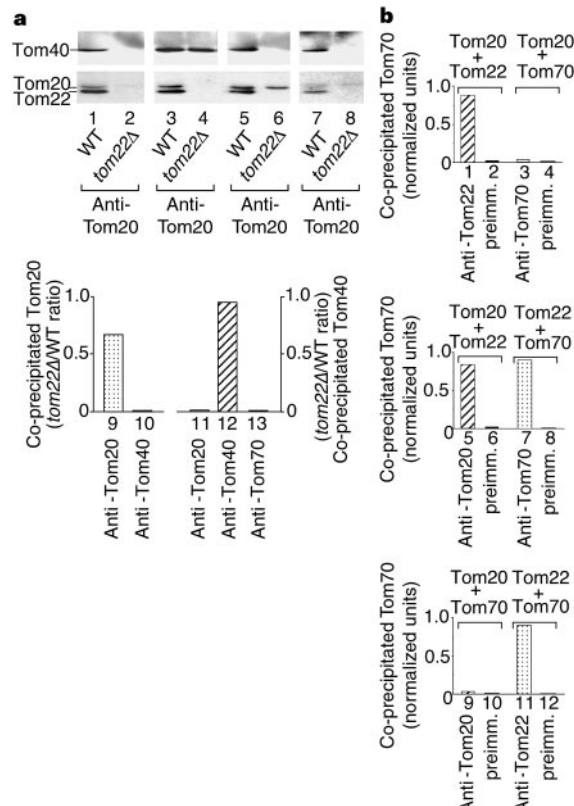


Figure 3 Tom22 as a docking point for Tom20 and Tom70. **a**, The lack of Tom22 inhibits the association of Tom20 and Tom70 with Tom40 (GIP). Digitonin-lysed wild-type and *tom22Δ* mitochondria were co-immunoprecipitated with antibodies against Tom22, Tom40, Tom20 or Tom70. The co-precipitated proteins were separated by SDS-PAGE and immunodecorated with antibodies against Tom40, Tom22 and Tom20. Precipitated Tom20 and Tom40 were quantified and the ratio of *tom22Δ* to wild type is shown. **b**, Interaction of the cytosolic domain of Tom22 with those of Tom20 and Tom70. The purified domains were mixed pairwise, followed by co-precipitation with antibodies against Tom20, Tom22, Tom70 or preimmune antibodies. The amount of direct precipitation of each domain with its genuine antibody was set to 1 (normalized units).

against the abundant porin did not precipitate crosslinking products of AAC–DHFR (Fig. 2d, lanes 5 and 10), confirming the specificity of the approach.

We investigated whether the peripheral receptors Tom70 and Tom20 partly substitute for the lacking receptor function of Tom22. Pretreatment of mitochondria with trypsin removes the cytosolic domains of all receptors and protein import is reduced in wild-type mitochondria to around 30% (bypass import)^{18,19} (Fig. 2e). With *tom22Δ* mitochondria, however, trypsin pretreatment only slightly affected the residual import (Fig. 2e). This result surprisingly indicates that, without Tom22, preprotein import may be largely independent of the typical receptor proteins, leaving the trypsin-resistant Tom5 as the only binding site for preproteins before insertion into the general import pore (GIP)²⁰.

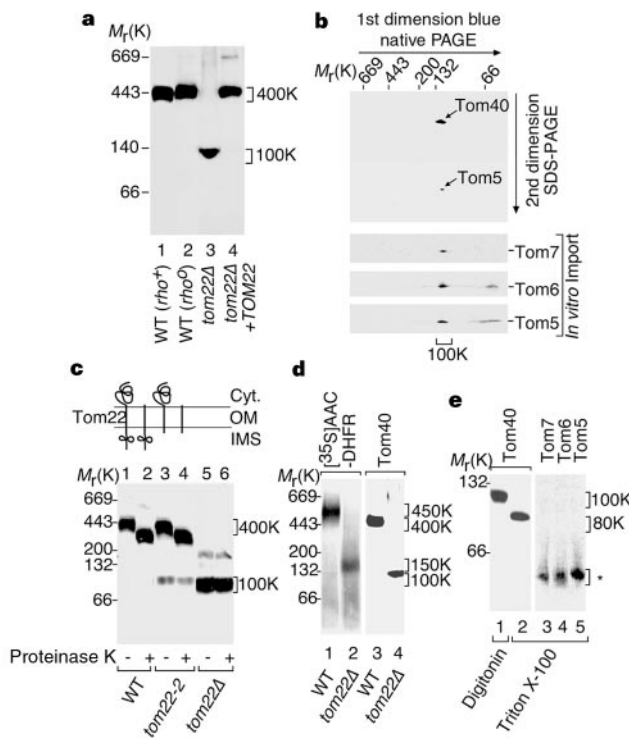


Figure 4 Lack of Tom22 causes dissociation of the 400K GIP complex. **a**, Dissociation to a 100K complex and restoration by re-expression of Tom22. Mitochondria were isolated from wild-type yeast (*rho*⁺), wild-type (*rho*⁰), *tom22Δ* and a *tom22Δ* strain re-expressing Tom22. Samples were lysed in digitonin, subjected to blue native PAGE and immunodecorated with anti-Tom40. **b**, Tom40, Tom7, Tom6 and Tom5 are in the 100K complex. *tom22Δ* mitochondria were lysed in digitonin, then separated by blue native PAGE and SDS-PAGE. Immunodecoration used anti-Tom40 and anti-Tom5. ³⁵S-labelled Tom7, Tom6 and Tom5 were imported into *tom22Δ* mitochondria and analysed by two-dimensional PAGE and autoradiography. **c**, The membrane-spanning segment of Tom22 is required to stabilize the 400K GIP complex. Mitochondria were isolated from wild-type yeast, the *tom22-2* strain expressing Tom22 without its intermembrane space (IMS) domain, and the *tom22Δ* strain. Mitochondria were treated with or without proteinase K, lysed in digitonin and analysed by blue native PAGE. Cyt, cytosolic side; OM, outer membrane. **d**, Accumulation of preprotein in the 100K subcomplex of *tom22Δ* mitochondria. ³⁵S-labelled AAC–DHFR was precubated with methotrexate and imported into wild-type and *tom22Δ* mitochondria. Mitochondria were lysed in digitonin and analysed by blue native PAGE and autoradiography (lanes 1 and 2). For lanes 3 and 4, digitonin-lysed mitochondria were analysed by blue native PAGE and immunodecoration for Tom40. **e**, Tom40 is present as a dimer in the 100K complex. ³⁵S-labelled Tom7, Tom6 and Tom5 were imported into *tom22Δ* mitochondria. Mitochondria were lysed with Triton X-100 (lanes 2–5) or digitonin (lane 1) and separated by blue native PAGE. Analysis was by immunodecoration for Tom40 (lanes 1 and 2) or autoradiography (lanes 3–5). Asterisk, position of small Toms close to the front of the gel.

It is possible that Tom22 is required for the docking of Tom20 and Tom70 to the GIP complex. The peripheral association of Tom20 and Tom70 with the GIP complex (Tom40) can be assayed by co-immunoprecipitation experiments under mild conditions^{18,21,22}. Antibodies against Tom40 co-precipitated Tom20 from wild-type mitochondrial lysates, but not from *tom22Δ* mitochondria (Fig. 3a, lanes 3 and 4, column 10). Similarly, anti-Tom20 did not precipitate Tom40 from *tom22Δ* mitochondria (Fig. 3a, lane 6, column 11). Moreover, the precipitation of Tom40 with anti-Tom70 was blocked with *tom22Δ* mitochondria (Fig. 3a, lane 8, column 13). The specificity of the co-immunoprecipitation approach is underscored by the lack of precipitation of Tom40 or Tom20 by anti-Tom22 from *tom22Δ* mitochondria (Fig. 3a, lane 2). When TOM22 is re-expressed in the *tom22Δ* strain, the association of Tom20 and Tom70 with Tom40 was restored to wild-type levels (not shown). Thus, the lack of Tom22 strongly reduces the interaction of the receptors Tom20 and Tom70 with the GIP.

To determine whether Tom22 directly interacts with Tom20 or Tom70, we assayed the interaction between the expressed and purified cytosolic domains of the receptors¹⁰. The cytosolic domains of Tom20 and Tom22 were mixed and subjected to co-immunoprecipitation. Tom20 was co-precipitated with anti-Tom22 (Fig. 3b, column 1), whereas no co-precipitation was observed with pre-immune serum (Fig. 3b, column 2) or when Tom22 was omitted from the incubation (not shown). Moreover, Tom22 was co-precipitated with anti-Tom20 (Fig. 3b, column 5). Similarly, Tom22 and Tom70 could be co-precipitated (Fig. 3b, columns 7 and 11). However, when Tom20 and Tom70 were mixed, only weak co-precipitation was observed (Fig. 3b, columns 3 and 9), demonstrating the selectivity of the association with Tom22. The previously reported transient interactions between Tom20 and Tom70

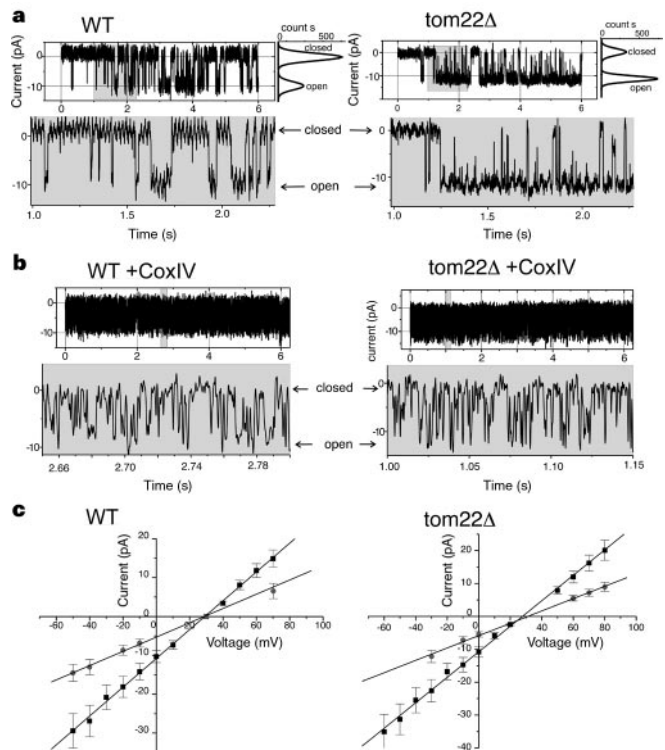


Figure 5 Increased open probability of the import channel of *tom22Δ* mitochondria. **a**, Current traces from a bilayer fused with wild-type or *tom22Δ* outer-membrane vesicles at a membrane potential of 0 mV. Bottom traces show time-scale-expanded current recordings from the top traces with high time resolution (10 kHz). **b**, Current traces (as in **a**) after adding 10 μM CoxIV(1–23) to the *cis* compartment. **c**, Current–voltage relation (without presequence peptide) of the fully open single channel (squares) and the most frequent subconductance level (circles).

are apparently less stable and involve smaller fractions of the proteins²³ so they were not detected by our co-precipitation approach. We conclude that the purified cytosolic domain of Tom22 can associate with the cytosolic domains of Tom20 and Tom70.

In wild-type mitochondria, Tom22, Tom40 and the three small proteins Tom5, Tom6 and Tom7 form the centre of the TOM machinery, the GIP complex of relative molecular mass 400,000 (M_r 400K) that remains stable on blue native electrophoresis, while the peripheral subunits Tom20 and Tom70 are mainly released^{13,20}. This complex was also observed in mitochondria from a *rho*⁰ strain (Fig. 4a, lanes 1 and 2). With *tom22Δ* mitochondria, however, the 400K GIP complex, detected with anti-Tom40, completely dissociated to a complex of ~100K (Fig. 4a, lane 3). Mitochondria isolated from the *tom22Δ* strain re-expressing Tom22 from a plasmid contained a large GIP complex (Fig. 4a, lane 4), demonstrating that the dissociation of the GIP complex was selectively caused by the lack of Tom22. By two-dimensional electrophoresis of *tom22Δ* mitochondria and subsequent immunodecoration, Tom40 and Tom5 were quantitatively found in the 100K complex (Fig. 4b, top). The preproteins of Tom7 and Tom6, for which specific antibodies are not available, were synthesized and ³⁵S-labelled *in vitro* and imported into the mitochondria. The bulk of Tom7 and Tom6 migrated at the 100K position (Fig. 4b, bottom), but a small amount of Tom6 was not assembled, as similarly observed with *in vitro* imported Tom5 (Fig. 4b, bottom)¹³. We conclude that the lack of Tom22 causes dissociation of the GIP complex to a 100K complex containing Tom40, Tom7, Tom6 and Tom5.

We then wondered which part of Tom22 is required for the integrity of the 400K GIP complex. Treatment of wild-type mitochondria with protease removed the cytosolic domains of the Tom22 molecules in the GIP complex, causing a shift of the GIP complex to ~350K but not to 100K (Fig. 4c, lane 2)¹³. The GIP complex of *tom22-2* mutant mitochondria, lacking the Tom22 intermembrane space domain⁹, preferentially migrated as a large complex (Fig. 4c, lane 3). When we removed both domains by treating *tom22-2* mitochondria with protease, we still observed a large GIP complex and very little 100K complex (Fig. 4c, lane 4). We conclude that neither the cytosolic domain nor the intermembrane space domain of Tom22 is required to maintain the stability of the bulk of GIP complexes. When the membrane anchor of Tom22 was removed (*tom22Δ* mitochondria), the GIP complex quantitatively dissociated and the vast majority of Tom40 was found at the 100K position (Fig. 4c, lane 5).

To determine whether the 100K complex retained a functional protein-import site, we arrested ³⁵S-labelled AAC-DHFR across the outer membrane. The intermediate accumulated at ~450K in wild-type mitochondria (Fig. 4d, lane 1) and at 150K in *tom22Δ* mitochondria (Fig. 4d, lane 2). The fusion protein is ~50K, indicating an accumulation in the 400K and 100K complexes, respectively. The 100K subcomplex therefore forms a functional import site. The wild-type TOM complex contains about six molecules of Tom40 (ref. 13) and, as suggested by electron micrographic analysis in *N. crassa*, up to three import channels²⁴, implying that a single import channel is formed by a dimer of Tom40. To test whether the 100K subcomplex fulfilled this prediction, we used Triton X-100 to release the small TOM proteins from Tom40 (Fig. 4e, lanes 3–5)¹³. Tom40, now the only component of the complex, migrated at 80K (Fig. 4e, lane 2). Monomeric Tom40, obtained after lysis of mitochondria with SDS, migrates at 40K to 45K (not shown). We propose that a dimer of Tom40 present in the 100K subcomplex forms the basic unit of one import channel.

To determine the electrophysiological characteristics of the translocase channel in *tom22Δ* outer membranes, we isolated outer-membrane vesicles from wild-type and *tom22Δ* mitochondria and analysed them using the planar lipid-bilayer technique¹². A single type of cation channel was observed in both membranes

(Fig. 5a). We investigated the channel's conductance, selectivity and sensitivity to the synthetic mitochondrial presequence of cytochrome oxidase subunit IV (CoxIV). The wild-type channel revealed a reversal potential (E_{rev}) of +30 mV ($P_{K^+}/P_{Cl^-} = 4.4 : 1$), its slope conductance was $\Lambda = 370 \pm 5$ pS for the main conductance and $\Lambda_1 = 180 \pm 6$ pS for the most frequent subconductant level (Fig. 5c, left). The channel of *tom22Δ* outer membranes revealed similar values: E_{rev} was +29 mV with a slope conductance of $\Lambda = 387 \pm 6$ pS and $\Lambda_1 = 187 \pm 5$ pS for the main conductance and the most frequent subconductant level, respectively (Fig. 5c, right). As observed with purified Tom40 (ref. 12), the direct transitions between the conductance levels and the comparatively high frequency of the states indicate that they represent distinct conductance states of a single channel, rather than the coupled opening of two or more individual channels. In support of this view, the ratio between the frequency of the main conductance state and that of the subconductance state did not change significantly between wild-type and *tom22Δ* outer membranes. The channels from both sources were highly sensitive to the presence of the CoxIV presequence peptide. The peptide strongly increased the frequency of channel gating (flickering) of both the wild-type channel and the *tom22Δ* channel (Fig. 5b). This effect was reversible by removal of the peptide. Thus, the basic properties of the channel are identical for wild-type outer membranes, *tom22Δ* outer membranes and purified Tom40 (ref. 12). However, a difference was observed for the open probability of the channel: in wild-type outer membranes, the channel was mainly in the closed state (Fig. 5a, left), whereas the channel of *tom22Δ* outer membranes was mainly in the open state (Fig. 5a, right), resembling that observed with purified Tom40 (ref. 12). We conclude that Tom22 is required to negatively regulate the open probability of the Tom40 channel.

Our results point to a new principle of organization of a multi-subunit preprotein translocase. The multidomain protein Tom22 not only functions as a receptor and *trans* binding site for preproteins, but it also organizes the interaction between the channel and receptor subcomplexes at two levels. The following functions can be assigned to the three domains of Tom22: the cytosolic domain plays a dual role, specifically recognizing preproteins^{6,8,10} and serving as a docking point for the peripheral receptors Tom20 and Tom70; the intermembrane space domain provides a *trans* binding site for presequences^{7,9}; and the single membrane anchor of Tom22 is crucial for the integrity of the GIP complex. In the absence of this membrane anchor, the GIP complex dissociates into small core complexes containing a dimer of Tom40 and the three small Toms. Such a 100K core complex probably contains a single channel that retains the basic channel properties but is already open in the absence of preproteins. In contrast, in the presence of Tom22, the wild-type GIP complex contains tightly regulated channels (probably three channels²⁴) that open when preproteins are present. Tom22 apparently represents a component of the machinery that controls the gate. Tom40 is the only strictly essential protein of the outer membrane translocase (cells lacking Tom40 were non-viable with all methods of gene deletion applied^{14,25} (not shown)) and forms the basic import core, whereas Tom22 is crucial for the organization of the core complexes into the large GIP complex, the interaction with the peripheral receptors, and the efficient and coordinated transfer of preproteins. Tom22 is therefore the central organizer that converts a simple channel into the complex, dynamic and efficiently coordinated machinery for the recognition and translocation of preproteins. □

Methods

Generation and analysis of *TOM22*-deficient yeast

A *Bam*HI/*Sal*I fragment containing the *TOM22* open reading frame was ligated into the high-copy vector pFL44 with the *MET25* promoter and the *CYC1* terminator, yielding the plasmid BG3036 (*URA3*, *TOM22*). This plasmid was transformed into the diploid strain

OL551 (*his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 ura3-52 ura3-52 trp1-Δ63/trp1-Δ3 TOM22/tom22::HIS3 rho⁺*) and then sporulated. The resulting haploid strain (*tom22::HIS3*) containing BG3036 was plated on medium containing 5-FOA; colonies growing at 28 °C were tested for the absence of the *TOM22* gene and one of them was designated OL201 (*his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 tom22::HIS3 rho⁺*). The lack of mitochondrial DNA in OL201 cells was demonstrated by crossing the cells with a *rho⁻* tester strain and by DAPI and HOECHST staining. The control haploid strain OL223 (*his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 rho⁺*) was generated by ethidium bromide mutagenesis of a *TOM22*-containing spore of OL551.

In vitro import of preproteins into mitochondria and crosslinking

³⁵S-labelled precursor proteins were synthesized in rabbit reticulocyte lysate¹⁹. The import assays included isolated yeast mitochondria and buffer (3% (w/v) BSA, 250 mM sucrose, 5 mM MgCl₂, 80 mM KCl, 10 mM MOPS/KOH, pH 7.2) in the presence of 2–4 mM ATP and 2–4 mM NADH at 25 °C. Phosphocreatine (10–20 mM) and phosphocreatine kinase (100–200 μg ml⁻¹) were added to allow efficient import into *rho⁰* mitochondria. After the import reaction, mitochondria were treated with 50 μg ml⁻¹ proteinase K for 15 min at 0 °C (refs 18, 21). The mitochondria were reisolated, washed and separated by SDS–PAGE. Pretreatment of mitochondria with trypsin or dissipation of the membrane potential was as described^{18,19}. For crosslinking, ³⁵S-labelled AAC–DHFR was accumulated at the outer mitochondrial membranes by adding 20 μM methotrexate to the import reaction. Mitochondria were reisolated and subjected to crosslinking with 0.4 mM EGS before immunoprecipitation under stringent conditions¹⁸.

Blue native electrophoresis and co-precipitation of Tom proteins

Mitochondrial pellets were lysed in 50 μl ice-cold digitonin buffer (1% (w/v) digitonin, 20 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% (v/v) glycerol, 1 mM PMSF) before the addition of sample buffer (5% (w/v) Coomassie brilliant blue G-250, 100 mM Bis–Tris, pH 7.0, 500 mM 6-aminocaproic acid). Blue native PAGE was performed using a 6–16.5% polyacrylamide gradient gel at 10 °C (ref. 13). For co-precipitation of Tom proteins from mitochondria, isolated mitochondria (250 μg of protein) were lysed in 0.5% digitonin-containing buffer and incubated with protein A–Sepharose to which antibodies were covalently coupled^{18,21,22}. After washing in digitonin-containing buffer, the bound proteins were analysed by SDS–PAGE and immunodecoration. For co-precipitation of purified Tom proteins, the expressed and purified cytosolic domains of Tom20, Tom22 or Tom70 (100 pmol each)¹⁰ were incubated pairwise in 50 mM KCl, 10 mM MOPS–KOH, pH 7.2, 0.5% BSA for 15 min at 25 °C (200 μl final volume). Aggregated material was removed by centrifugation for 20 min at 106,000g. The supernatant was incubated with anti-Tom antibodies coupled to protein A–Sepharose for 1 h at 4 °C. After two washes with 50 mM KCl, 10 mM MOPS–KOH, pH 7.2, the precipitate was analysed by SDS–PAGE, immunodecoration and fluorimaging (Fuji).

Electrophysiological measurements

Planar lipid bilayers were made by using the painting technique^{12,26}. After formation of a stable bilayer (*cis* chamber, 250 mM KCl, 10 mM CaCl₂, 10 mM MOPS/Tris, pH 7.0; *trans* chamber, 10 mM KCl, 10 mM MOPS/Tris, pH 7.0), outer-membrane vesicles were added to the *cis* chamber directly below the bilayer. All membrane potentials are referred to the *trans* compartment. Under these conditions, the porin channel was closed. The fusion rates of *tom22Δ* outer-membrane vesicles with the planar lipid bilayer were significantly higher than that of wild-type outer-membrane vesicles, supporting the idea of a higher open probability for the *tom22Δ* channel.

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Negative regulation of erythropoiesis by caspase-mediated cleavage of GATA-1

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The production of red blood cells follows the sequential formation of proerythroblasts and basophilic, polychromatophilic and orthochromatic erythroblasts, and is promoted by the hormone erythropoietin (Epo) in response to tissue hypoxia¹. However, little is known about the negative regulation of this process². Death receptors are a family of surface molecules that trigger caspase activation and apoptosis in a variety of cell types^{3–5}. Here we show that immature erythroid cells express several death receptors whose ligands are produced by mature erythroblasts. Exposure of erythroid progenitors to mature erythroblasts or death-receptor ligands resulted in caspase-mediated degradation of the transcription factor GATA-1, which is associated with

impaired erythroblast development. Expression of a caspase-resistant GATA-1 mutant, but not of the wild-type gene, completely restored erythroid expansion and differentiation following the triggering of death receptors, indicating that there is regulatory feedback between mature and immature erythroblasts through caspase-mediated cleavage of GATA-1. Similarly, erythropoiesis blockade following Epo deprivation was largely prevented by the expression of caspase-inhibitory proteins or caspase-resistant GATA-1 in erythroid progenitors. Caspase-mediated cleavage of GATA-1 may therefore represent an important negative control mechanism in erythropoiesis.

Human progenitor cells are refractory to the stimulation of death receptors during the initial phases of erythroid differentiation, but they later acquire a transient sensitivity to death receptors that persists until the polychromatophilic erythroblast stage². To determine whether the engagement of death receptors interferes with erythroid cell development, late erythroid progenitors undergoing Epo-driven differentiation were stimulated with an agonist anti-CD95 monoclonal antibody. CD95 crosslinking strongly inhibits erythroid differentiation as the generated cells are mostly blocked at the basophilic erythroblast stage (Fig. 1a). This antidifferentiative effect was abolished by adding the caspase inhibitor zVAD, indicating that active caspases are involved in differentiation arrest. In addition to CD95 (ref. 2), erythroblasts express other death receptors,

including tumour-necrosis factor (TNF) receptor I (ref. 6), DR4 (TRAIL receptor-1) and DR5 (TRAIL receptor-2) (Fig. 1b). Moreover, immunoblot and flow cytometry analysis showed that TRAIL (Apo-2 ligand), which is the ligand for DR4 and DR5, is expressed in mature erythroblasts (Fig. 1b, c). TRAIL and TNF- α displayed a consistent caspase-mediated antidifferentiative effect on immature erythroid cells (Fig. 1d), indicating that other death-receptor ligands may contribute with CD95L to the negative regulation of erythropoiesis. To distinguish the apoptotic from the antidifferentiative effects induced by death-receptor crosslinking, increasing amounts of anti-CD95 were added to erythroid cultures. Only a few immature erythroblasts underwent apoptosis when exposed to saturating concentrations of anti-CD95 (Fig. 1e), whereas lower concentrations of anti-CD95, unable to induce cell death, significantly block erythroid differentiation (Fig. 1e, f). For the rest of the study, we used the anti-CD95 dosage (100 ng ml⁻¹) effective for differentiation blockade but not for inducing detectable erythroblast apoptosis within one week of stimulation.

We next investigated whether caspase activation was compatible with erythroid cell survival. Although Epo deprivation results in caspase activation and immature erythroblast apoptosis (Fig. 2a, b), the presence of Epo completely prevented erythroid apoptosis but did not inhibit caspase-3-like activity following CD95 stimulation (Fig. 2a, b), indicating that Epo may block the death pathway by acting downstream of caspases. However, even in the absence of cell death, CD95-induced caspase activity considerably impaired erythroid cell expansion (Fig. 2c). Both growth and differentiation arrest were completely restored by removing anti-CD95 from the

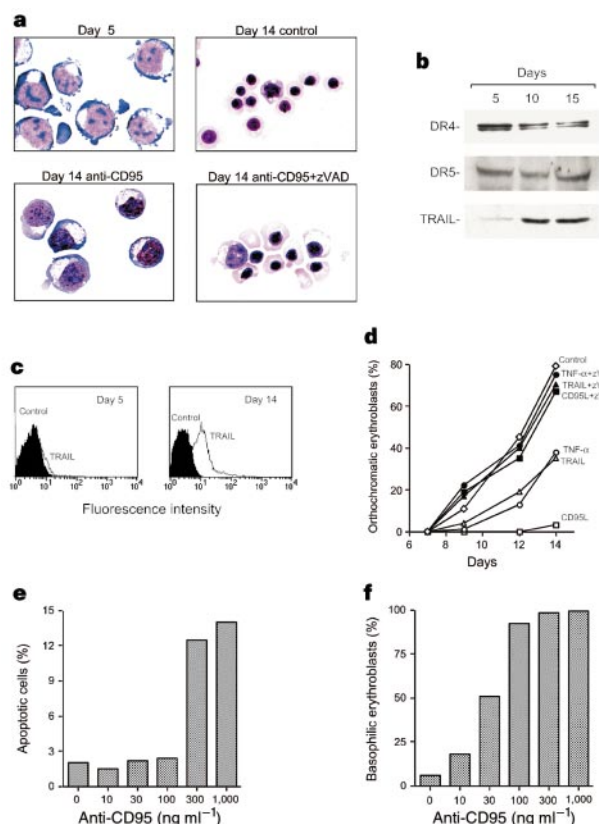


Figure 1 Death receptors trigger arrest of erythroid differentiation. **a**, CD34⁺ haematopoietic progenitors undergoing Epo-induced differentiation after 5 days of culture (top left) treated with control IgM (top right), anti-CD95 (bottom left), or anti-CD95 plus zVAD (bottom right), and examined for morphology on day 14. **b**, Immunoblot analysis of TRAIL and TRAIL receptors in erythroid cells at different maturation stages. **c**, Surface expression of TRAIL in late erythroid progenitors and in mature erythroblasts. **d**, Inhibitory effect of recombinant death-receptor ligands (100 ng ml⁻¹) on erythroblast development in the presence or in the absence of zVAD. **e**, **f**, Effect of different anti-CD95 concentrations on the apoptotic rate of day-7 erythroblasts treated for 48 h (**e**) and on erythroid differentiation after 14 days of culture (**f**).

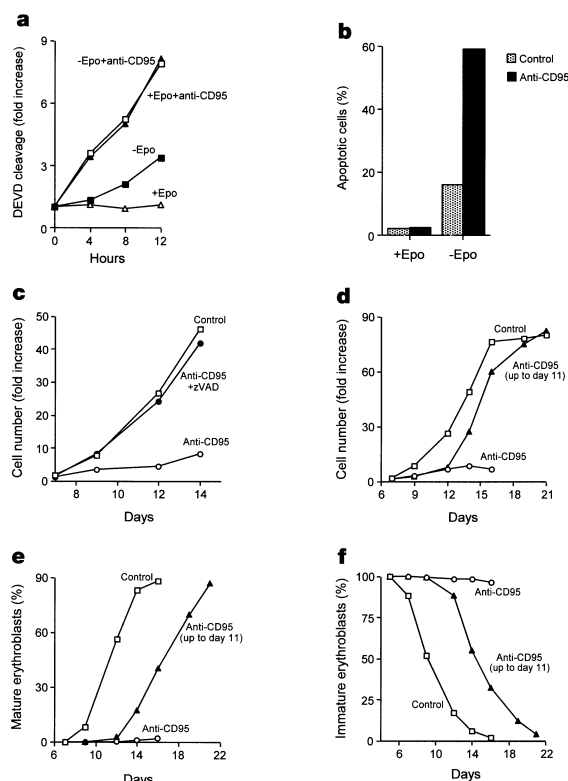


Figure 2 CD95-induced inhibition of erythropoiesis in the absence of cell death is reversible. **a**, **b**, Effect of Epo and anti-CD95 stimulation on caspase activity (**a**) and viability (**b**). **c**, **d**, Growth curve of erythroid cultures treated with control IgM, anti-CD95 or anti-CD95 plus zVAD. **e**, **f**, Differentiation curve evaluating mature (orthochromatic erythroblasts) (**e**) and immature (pro-erythroblasts and basophilic erythroblasts) erythroid cells (**f**) in the same cultures as **d**. A representative experiment of three performed is shown.

erythroid cultures (Fig. 2d–f), indicating that the triggering of death receptors may cause a temporary block of erythropoiesis.

The first two members of the GATA transcription factor family are important for erythroid differentiation⁷. Gene knockout studies have shown that GATA-1 is required for terminal differentiation of erythroid precursors⁸, which undergo developmental arrest and apoptosis when GATA-1 is absent⁹. Similarly, disruption of the gene encoding GATA-2 results in severe anaemia, leading to early embryonic death¹⁰. To test the idea that the arrest of CD95-induced erythroid differentiation may be due to altered levels of erythroid transcription factors, we evaluated the effect of CD95 stimulation on GATA-1 and GATA-2 expression in maturing erythroblasts. A dramatic decrease in GATA-1 expression was observed in erythroblasts treated for two days with anti-CD95 in the absence of zVAD (Fig. 3a, b), indicating that caspases mediate GATA-1 downregulation in these cells. For other transcription factors, GATA-2 increased in CD95-stimulated erythroblasts (Fig. 3a, b), whereas the amount

of PML and PU.1 was not affected and NF-E2 was significantly decreased (data not shown). The increase in GATA-2 levels is not surprising because GATA-2 is strongly upregulated in erythroid cells lacking GATA-1 (ref. 7). Although NF-E2 is not required for erythropoiesis, it is needed for megakaryocytic cell development¹¹, which is also promoted by GATA-1 (ref. 12). We therefore investigated whether CD95 triggering interferes with megakaryopoiesis. Anti-CD95 treatment impaired the differentiation of most megakaryocytic cells, which increased in number and underwent endomitosis much less efficiently than control cultures (Fig. 3c and results not shown); these are two key features observed in megakaryocytes lacking GATA-1 (ref. 12). As observed in erythroid cells, impaired differentiation in megakaryocytic cells was prevented by caspase inhibitors and was associated with caspase-mediated downregulation of GATA-1 and NF-E2 (Fig. 3c, d). Immature erythroid cells cultured with mature erythroblasts displayed a similar anti-differentiative effect coupled with GATA-1 downregulation, unless erythroid progenitors were retrovirally transduced with genes that block death-receptor signalling^{13–15} (Fig. 3e, f), indicating that the accumulation of mature erythroblasts may moderate the development of immature erythroid cells through death-receptor triggering and caspase activation.

Caspase activation results in proteolytic cleavage adjacent to the aspartic acid of proteins carrying caspase recognition motifs^{16–18}. Because caspase inhibitors restore both differentiation and GATA-1 in immature erythroblasts, we evaluated whether GATA-1 is targeted by caspases. *In vitro*-translated [³⁵S]methionine-labelled GATA-1 was incubated with purified caspases to identify potential cleavage sites. Several GATA-1 proteolytic fragments generated by exposure to the major caspases are shown in Fig. 4a, indicating that GATA-1 contains three potential cleavage sequences. These are two minor sites at EGLD at position 42 and LSPD at position 144, and a major site at EDLD at position 125, which is cleaved by caspase-3, -7, -8, -9 and -10, resulting in fragments of relative molecular mass 30,000 (*M_r* 30K) and 16,000 (16K). To determine whether GATA-1 is cleaved *in vivo* upon CD95 triggering, erythroblasts 7 days old were treated for 12 h with anti-CD95 and analysed by immunoblot for the presence of GATA-1 fragments, using an antibody specific for the carboxy terminus of the protein. Anti-CD95-activated caspases induced the formation of a 30K GATA-1 fragment (Fig. 4b), whereas

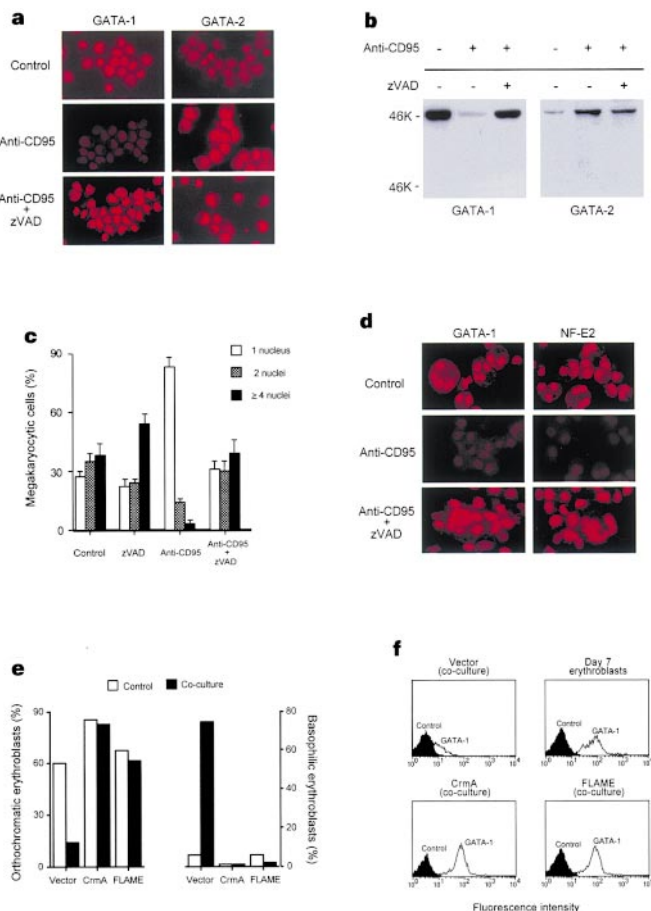


Figure 3 Anti-CD95 and mature erythroblasts induce GATA-1 downregulation and impaired erythroid differentiation. **a**, Immunofluorescence analysis for GATA-1 and GATA-2 of day-7 erythroblasts treated for 2 days with control IgM (top), anti-CD95 (middle) or anti-CD95 plus zVAD (bottom). **b**, Cells treated as in **a** were analysed by immunoblot for expression of GATA-1 and GATA-2. **c**, CD34⁺ haematopoietic progenitors undergoing thrombopoietin-induced differentiation were treated with control IgM (control), zVAD, anti-CD95, or anti-CD95 plus zVAD, and examined for morphology on day 12. Data are mean $\pm$ s.d. of five independent experiments. **d**, Immunofluorescence analysis for GATA-1 and NF-E2 of day-9 megakaryocytic cultures. **e**, **f**, Erythroid progenitors transduced with empty vector, CrmA or FLAME-1 were grown for 4 days with (co-culture) or without (control) a sixfold higher number of mature erythroblasts and analysed for morphology (**e**) and GATA-1 expression by flow cytometry (**f**) together with day-7 erythroblasts as a positive control. A representative experiment of three performed is shown.

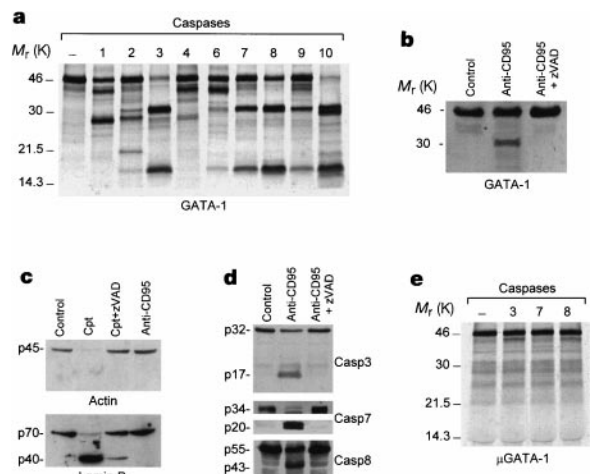


Figure 4 GATA-1 is a target of caspases *in vitro* and *in vivo*. **a**, *In vitro*-translated radiolabelled GATA-1 was exposed to purified caspases and analysed by SDS/PAGE. **b–d**, Immunoblot analysis of day-7 erythroblasts treated for 12 h with control IgM, anti-CD95, anti-CD95 plus zVAD, 500 ng ml⁻¹ camptothecin (Cpt) or Cpt plus zVAD. **e**, *In vitro*-translated radiolabelled μ GATA-1 (mutated at Asp 125 with Glu) was exposed to purified caspase-3, -7 and -8 and analysed as in **a**.

substrates such as actin and lamin B were spared unless cells were treated with lethal cytotoxic drugs (Fig. 4c). Thus, GATA-1 is a preferential caspase substrate in CD95-stimulated erythroblasts, cleaved by one or more of caspase-3, -7, -8, -9 and -10 at aspartic acid 125. Caspase-9 and caspase-10 were weakly and not expressed, respectively, in immature CD95-sensitive erythroblasts. In contrast, caspase-3, -7 and -8 were highly expressed and activated by CD95 triggering (Fig. 4d), so these are the most likely candidates for *in vivo* GATA-1 cleavage. A similar caspase-activation pattern was observed following stimulation with TNF- α and TRAIL (not shown). To obtain a GATA-1 protein resistant to caspase-mediated cleavage in erythroblasts, the aspartic acid at position 125 was mutated to glutamic acid. This GATA-1 mutant (μ GATA-1) was not cleaved *in vitro* by caspase-3, -7 or -8 (Fig. 4e). As for GATA-1, NF-E2 downmodulation in both erythroid and megakaryocytic cells is due to caspase-mediated cleavage at aspartic acid at position 73 (not shown).

We next investigated whether μ GATA-1 was resistant to caspase-mediated cleavage in erythroblasts. GATA-1 and μ GATA-1 were cloned in PINCO retroviral vector and transduced into cycling CD34⁺ cells¹⁹. These progenitors were then analysed by flow cytometry and sorted on the basis of comparable expression of the green fluorescent protein (GFP) reporter (Fig. 5a), which ensured that there was similar expression of exogenous GATA-1 and μ GATA-1 in the transduced cells¹⁹. Erythroid cells transduced with μ GATA-1, but not with wild-type GATA-1, expressed high levels of this transcription factor following treatment with anti-CD95 in the absence of zVAD (Fig. 5b), indicating that μ GATA-1 was resistant *in vivo* to caspase-mediated degradation. Moreover, expression of μ GATA-1 in erythroblasts abolished the expansion

and differentiation arrest induced by anti-CD95, whereas wild-type GATA-1 was largely unable to overcome the erythropoietic blockade (Fig. 5c, d). Thus, caspase-mediated cleavage of GATA-1 underlies the impaired erythroid differentiation triggered by CD95.

The amount of Epo regulates the production of red blood cells¹. We examined the ability of a dominant-negative mutant of caspase-8 (C8DN) and the cowpox-virus protein CrmA, an effective inhibitor of caspase-1 and caspase-8 (ref. 13) to promote the survival and differentiation of erythroid progenitor cells in the absence of Epo. Erythroid progenitors expressing either CrmA or C8DN maintained large amounts of GATA-1 and differentiated to Epo-independent orthochromatic erythroblasts, whereas most of the empty-vector-transduced cells died or failed to differentiate (Fig. 5e, f). Again, this anti-erythropoietic role of caspases seems to be mediated at least in part by the cleavage of GATA-1, because μ GATA-1-transduced progenitors can largely survive and differentiate after early Epo deprivation (Fig. 5e) by preventing massive GATA-1 downmodulation (Fig. 5f).

Our data show that the interaction between death receptors and their ligands results in caspase-mediated degradation of GATA-1 and blockade of erythropoiesis. In physiological conditions, the accumulation in the erythroblastic island of mature erythroblasts expressing death-receptor ligands may temporarily inhibit the expansion and differentiation of immature erythroblasts sensitive to death receptors. Because the level of caspase activation is related to the intensity of death-receptor stimulation, the degree of early erythroblast inhibition may relate to the number of mature erythroblasts in the island. Moreover, we have shown that caspase activation contributes to the contraction of erythropoiesis observed after Epo deprivation. The decreased production of red blood cells that follows a reduction in Epo levels may result at least in part from caspase-8-mediated cleavage of GATA-1, as erythroid progenitors expressing μ GATA-1 or a dominant-negative mutant caspase-8 tend to lose Epo dependency. Although the role of caspase-8 in embryonic erythroid development is still unclear, our results may help explain the phenotype of caspase-8 knockout embryos, which exhibit congested erythrocyte accumulation in most blood vessels and in many organs²⁰.

The effects of GATA-1 cleavage in immature erythroblasts are similar to those observed in GATA-1-negative erythroid progenitors, which undergo differentiation arrest at the proerythroblast stage and die by apoptosis⁹. Indeed, prolonged analysis of death-receptor-triggered erythroblasts confirmed that GATA-1 downmodulation prevents the long-term survival of erythroid cells, indicating that this mechanism may be involved in dyserythropoiesis owing to persistent death-receptor stimulation, as reported in rheumatoid arthritis²¹, in myelodysplastic syndromes²², and possibly in aplastic anaemia^{23,24}.

We propose that caspases bring about the arrest of erythroid differentiation through cleavage of GATA-1. This mechanism may underlie the inhibition of erythropoiesis in a variety of physiological and pathological processes.

Methods

Antibodies

Antibodies against GATA-1 (N1 and M20), GATA-2 (CG2-96), lamin B (C20) and TRAIL (K18, for flow cytometry) were used (Santa Cruz). Anti-DR4/TRAIL-R1 and anti-DR5/TRAIL-R2 antibodies were from Biosource International (Camarillo, CA); anti-actin was from Sigma. Anti-caspase-3 and anti-TRAIL (B35-1, for immunoblot) antibodies were from PharMingen and anti-caspase-8 and agonist anti-CD95 (clone CH-11) monoclonal antibodies were from Upstate Biotechnology (Lake Placid, NY).

Liquid suspension culture

Purified human haematopoietic progenitors were grown in serum-free medium supplemented with 0.01 U ml⁻¹ interleukin-3, 0.001 ng ml⁻¹ GM-CSF, and 3 U ml⁻¹ Epo for erythroid cultures, or 100 ng ml⁻¹ thrombopoietin for megakaryocytic cultures²⁵. Morphological data were expressed as percentages of total cell number. The number of apoptotic cells was evaluated as described².

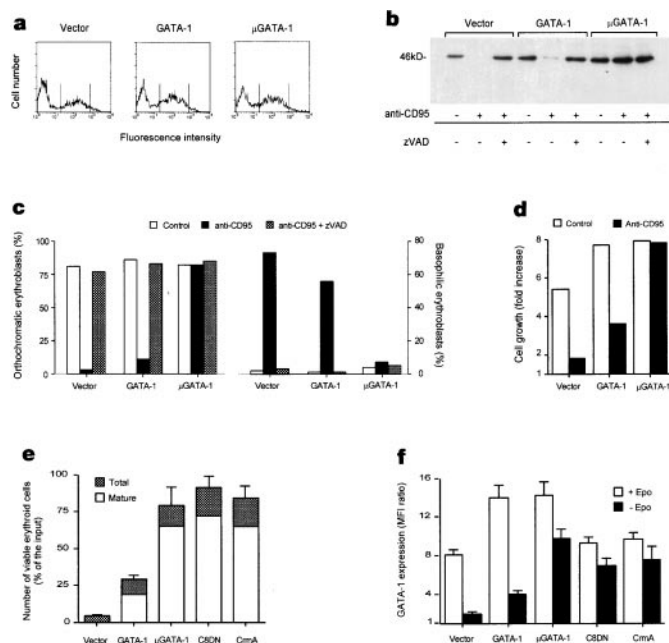


Figure 5 Blockade of GATA-1 degradation protects late erythroid progenitors from death and differentiation arrest. **a**, Flow-cytometry profiles of CD34⁺ haematopoietic progenitors transduced with empty PINCO vector, wild-type GATA-1 or Asp 125-mutated GATA-1 (μ GATA-1). More than 98% of sorted cells were included between the indicated sorter markers. **b**, Immunoblot analysis for GATA-1 expression in day-7 erythroid cells (cultured 4 days with cycling growth factors and 3 days with erythroid standard medium) transduced as in **a**, and treated for 2 days with or without anti-CD95 and zVAD. **c, d**, Effect of μ GATA-1 expression on erythroid progenitor differentiation (**c**) and expansion (**d**) after 6 days of CD95 stimulation. **e, f**, Viability and differentiation (**e**) and GATA-1 (**f**) of day-6 erythroid cells cultivated for 7 days (**e**) or 2 days (**f**) in the absence of Epo. MFI, mean fluorescence index. Data are mean $\pm$ s.d. of three independent experiments.

Immunostaining and flow-cytometry analysis

Cells were smeared on glass slides by cytocentrifugation, stained as described²⁶ and analysed by epifluorescence microscopy. Alternatively, cells were fixed in 2% paraformaldehyde, permeabilized in 0.05% Triton, incubated with anti-GATA-1 or control antibody, stained with phycoerythrin-labelled secondary antibodies and analysed by flow cytometry. Data were expressed as mean fluorescence index ratio between anti-GATA-1 and control antibody values.

Expression of caspases in bacteria and GATA-1 cleavage assay

Complementary DNAs for human caspases were subcloned in bacterial expression vectors of the pET21 series in frame with amino-terminal T7 tag and a carboxy-terminal six-histidine tag, as described²⁷. Proteins were expressed in BL21 (DE3) bacteria, purified on a Ni²⁺ affinity resin, assayed for efficient activity on specific fluorogenic peptides, and used for *in vitro* cleavage assay. GATA-1 translation reaction (1 µl) was incubated with 2 µl purified caspases in ICE buffer (25 mM Hepes, 1 mM EDTA, 5 mM DTT, 0.1% CHAPS, pH 7.5) in a final volume of 10 µl. The reaction was incubated for 1 hour at 37 °C and analysed by Tricine-SDS/PAGE and autoradiography.

Immunoblot and caspase activation assay

For detection of actin and caspases, cells were lysed in 1% NP40 lysis buffer in the presence of 1 mM phenylmethylsulphonyl fluoride and 2 µg ml⁻¹ each of aprotinin, leupeptin and pepstatin. The cell extracts for detection of GATA-1, GATA-2 and lamin B were made with 2% SDS lysis buffer. Proteins were analysed by standard immunoblot procedure. Caspase activity was evaluated by DEVD cleavage in a fluorogenic assay as described².

Mutagenesis and *in vitro* transcription or translation

Potential aspartate processing sites of GATA-1 and the catalytic cysteine of caspase-8 were mutated to glutamate and alanine by site-directed mutagenesis, using overlapping polymerase chain reaction (PCR) mutagenic oligonucleotides to produce caspase-resistant GATA-1 and a dominant-negative mutant of caspase-8 (ref. 28). Wild-type and mutated cDNAs were transcribed *in vitro* and translated in the presence of [³⁵S]methionine.

Production of retroviral particles and infection of haematopoietic progenitors

FLAME-1, CrmA, C8DN, wild-type and mutated GATA-1 cDNAs were cloned into the PINCO vector¹⁹. Culture supernatants containing viral particles were collected at 48 h after the amphotropic packaging cell-line transfection, and CD34⁺ cells exposed to cycling growth factors were infected¹⁹. GFP-positive cells were sorted 24 h after the last infection cycle using a FACS VANTAGE cell sorter (Becton Dickinson)¹⁹.

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Cytochrome P450 2C is an EDHF synthase in coronary arteries

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In most arterial beds a significant endothelium-dependent dilation to various stimuli persists even after inhibition of nitric oxide synthase and cyclo-oxygenase. This dilator response is preceded by an endothelium-dependent hyperpolarization of vascular smooth muscle cells, which is sensitive to a combination of the calcium-dependent potassium-channel inhibitors charybdotoxin and apamin, and is assumed to be mediated by an unidentified endothelium-derived hyperpolarizing factor (EDHF)^{1,2}. Here we show that the induction of cytochrome P450 (CYP) 2C8/34 in native porcine coronary artery endothelial cells by β-naphthoflavone enhances the formation of 11,12-epoxyeicosatrienoic acid, as well as EDHF-mediated hyperpolarization and relaxation. Transfection of coronary arteries with CYP 2C8/34 antisense oligonucleotides results in decreased levels of CYP 2C and attenuates EDHF-mediated vascular responses. Thus, a CYP-epoxygenase product is an essential component of EDHF-mediated relaxation in the porcine coronary artery, and CYP 2C8/34 fulfils the criteria for the coronary EDHF synthase.

The term EDHF is likely to comprise several factors, as the pharmacological characteristics of the nitric oxide synthase/cyclo-oxygenase (NOS/COX)-inhibitor-insensitive, endothelium-dependent hyperpolarization in different arteries and species are clearly distinct³. Although the EDHF that mediates relaxation in many vascular beds remains to be identified, there is indirect evidence that a CYP-epoxygenase-derived product mediates NOS/COX-independent relaxation in the human⁴, porcine^{5,6}, bovine^{5,7}, canine^{8,9} and rat^{10,11} coronary circulations. Indeed, 11,12-, 14,15-, 8,9- and 5,6-epoxyeicosatrienoic acids (EETs) and their dihydroxyeicosatrienoic acid derivatives both hyperpolarize and relax coronary vascular

smooth muscle cells^{7,9,12}. Moreover, the P450 inducer β -naphthoflavone can enhance smooth muscle hyperpolarization elicited by the putative CYP-dependent EDHF⁶. A role for an endothelium-derived CYP product in agonist-induced vascular relaxation was proposed over a decade ago, on the basis of the observation that inhibition of phospholipase A₂ and CYP attenuates the transient endothelium-dependent relaxation of isolated canine arteries¹. CYP inhibitors have since been shown to attenuate EDHF-mediated hyperpolarization and relaxation^{4,11–13}, but these compounds are not isoform-selective and can directly affect the activity of calcium-dependent potassium (K_{Ca}⁺) channels^{14,15}. Thus, an alternative, non-pharmacological approach is required to elucidate the role of a CYP enzyme in EDHF-mediated vascular responses.

To determine which CYP isoform could be implicated in the generation of EDHF-mediated responses we used polymerase chain reaction with reverse transcription (RT-PCR) to screen primary cultures of porcine aortic and human umbilical vein endothelial cells, as well as human coronary artery and lung microvascular endothelial cells. Of the CYP epoxygenases detected, 1A, 2C and 2J

were expressed in porcine aortic and human umbilical vein endothelial cells, and 2C and 2J were also expressed in lung microvascular and coronary artery endothelial cells. Only the globally expressed CYP 2C family, and more specifically the human CYP 2C8 (homologous to the porcine CYP 2C34) fitted the criteria for an EDHF synthase, as its expression was consistently enhanced by β -naphthoflavone in all of the cultured endothelial cell types studied (not shown). Other CYP 2C isoforms of human origin are 2C9, 10, 17, 18 and 19. Of these, CYP 2C9 is an arachidonic acid epoxygenase which produces EETs and, although it is highly expressed in human liver, we could not identify this isoform by RT-PCR in any of the human endothelial cells tested. Neither CYP 2C17 (a splice variant of 2C18 and 2C19) nor 2C18 were detectable in any of the human endothelial cells and although CYP 2C19 was detectable, its expression was unaffected by β -naphthoflavone. Note, however, that CYP 2C18 and 2C19 are not reported to exhibit any epoxygenase activity¹⁶.

To establish a functional link between the expression of CYP 2C8/34 and the production of EDHF, experiments were performed using isolated endothelium-intact porcine coronary arteries (PCA). Intraluminal incubation with β -naphthoflavone enhanced the expression of CYP 2C messenger RNA and protein in native coronary artery endothelial cells (Fig. 1a, b). This induction of CYP 2C resulted in a leftward shift in the EDHF-mediated concentration-relaxation curve to bradykinin ($EC_{50} = -6.18 \pm 0.33$ M in control versus -7.53 ± 0.40 M in β -naphthoflavone-treated rings, $P < 0.05$, $n = 4$; Fig. 1d), and potentiated the bradykinin-induced, EDHF-mediated hyperpolarization of PCA (Fig. 2a). No CYP 2C signal was detected in endothelium-denuded PCA. β -naphthoflavone did not affect the resting membrane potential of smooth

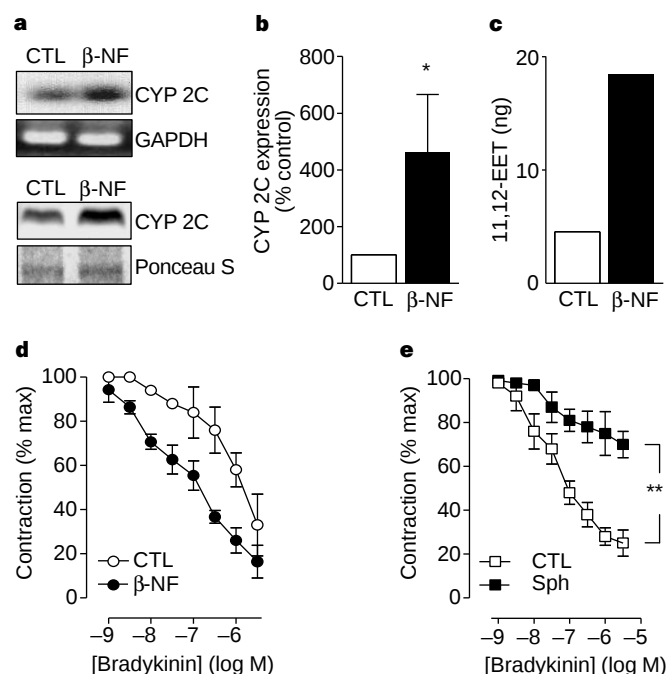


Figure 1 Increased expression of CYP 2C enhances EDHF-mediated relaxation of PCA and generation of 11,12-EET. PCA were perfused with culture medium containing either solvent (CTL; 48 h) or β -naphthoflavone (β -NF; 3 μ M, 48 h). **a**, Total RNA, harvested by intraluminal incubation of PCA with guanidine isothiocyanate, was analysed by RT-PCR followed by Southern blotting with CYP 2C and GAPDH probes. Total cellular phenol-soluble proteins were analysed by western blotting with a polyclonal CYP 2C antibody. Autoradiograph is representative of three additional experiments; Ponceau S staining shows equal loading of each lane of the protein gel. **b**, Statistical summary (mean $\pm$ s.e.m., $n = 4$, $*P < 0.05$) of the β -naphthoflavone-induced increase in CYP 2C. **c**, Amount of 11,12-EET determined by HPLC using endothelial cell extracts obtained following intraluminal incubation of coronary arteries with either solvent (48 h) or β -naphthoflavone (3 μ M, 48 h). The results are presented as the median of duplicate samples; similar results were obtained in two additional experiments. **d**, PCA incubated with either solvent (48 h) or β -naphthoflavone (3 μ M, 48 h) were cut into rings and preconstricted with U46619 (0.1–1 μ M), and the EDHF-mediated relaxation to bradykinin was determined in the presence of diclofenac (10 μ M) and N^o-nitro-L-arginine (300 μ M). Results are presented as the mean $\pm$ s.e.m. of 4 separate experiments. **e**, PCA were cut into rings and preconstricted with U46619 (0.1–1 μ M), and the EDHF-mediated relaxation to bradykinin was determined in the presence of diclofenac (10 μ M) and N^o-nitro-L-arginine (300 μ M), with or without sulphaphenazole (Sph; 10 μ M). Results are presented as the mean $\pm$ s.e.m. of 6 separate experiments; $**P < 0.01$ versus control.

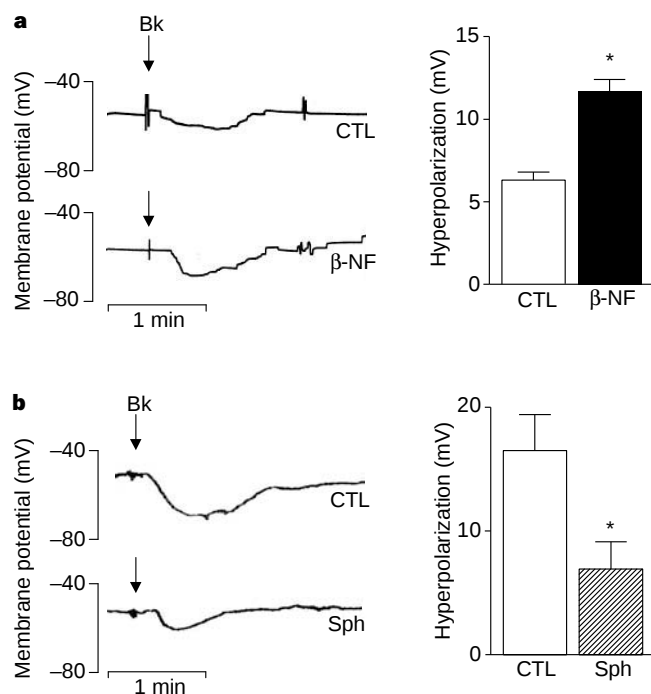


Figure 2 Increased expression of CYP 2C enhances, and sulphaphenazole inhibits, the EDHF-mediated hyperpolarization of PCA. The bradykinin (Bk; 100 nM)-induced hyperpolarization was determined in endothelium-intact PCA segments. Coronary artery segments were pretreated with **a**, solvent (CTL; 48 h) or β -naphthoflavone (β -NF; 3 μ M, 48 h), or **b**, solvent or sulphaphenazole (Sph; 10 μ M, 30 min). All experiments were performed in the presence of diclofenac (10 μ M), N^o-nitro-L-arginine (300 μ M) and U46619 (1 μ M). Left, representative membrane-potential recordings; right, statistical summary of data obtained in 4–6 separate experiments; $*P < 0.05$ versus control.

muscle cells (50 ± 1.8 mV in control versus 51.5 ± 2.1 mV in β -naphthoflavone-treated PCA) or the hyperpolarization elicited by 11,12-EET (not shown). Sulphaphenazole, which selectively inhibits CYP 2C8/9 in human liver¹⁷, significantly attenuated the bradykinin-induced EDHF-mediated relaxation of PCA rings (Fig. 1e) as well as EDHF-induced hyperpolarization (Fig. 2b), without affecting 11,12-EET-induced hyperpolarization (not shown).

CYP-derived metabolites of arachidonic acid synthesized in endothelial cells harvested from PCA were analysed by high-performance liquid chromatography (HPLC)¹⁸. Endothelial cells maintained under control conditions for 48 h generated 11,12-EET, and incubation with β -naphthoflavone induced a 3.8-fold increase in 11,12-EET generation (Fig. 1c). In some, but not all, β -naphthoflavone-treated endothelial cells 8,9-EET was also found, whereas 14,15- and 5,6-EET were not detected.

Although bradykinin-induced hyperpolarization of PCA was detectable for up to 60 h, the amplitude of the hyperpolarization decreased in a time-dependent manner upon incubation in culture medium, with hyperpolarizations of 17.3 ± 0.8 , 8.6 ± 0.9 and 6.3 ± 0.5 mV being obtained after 6, 20 and 48 h, respectively ($P < 0.05$, $n = 8$). There was a corresponding shift to the right in the bradykinin-induced EDHF-mediated relaxation of PCA incubated for various times (compare Fig. 1e and 1d). In all experiments, the combination of charybdotoxin and apamin completely inhibited EDHF-induced hyperpolarization and relaxation (not shown). The combined application of barium and ouabain, which inhibits CYP-independent, EDHF-mediated hyperpolarization in the rat hepatic and mesenteric arteries¹⁹, failed to affect bradykinin-induced, EDHF-mediated vascular responses (not shown).

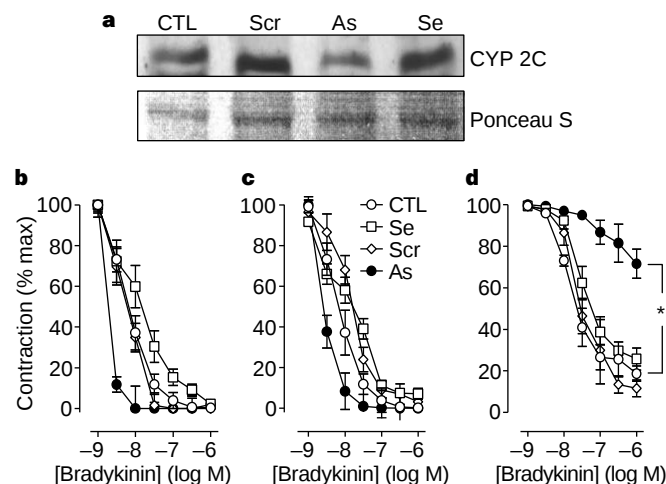


Figure 3 CYP 2C8/34 antisense oligonucleotides attenuate the expression of CYP 2C protein and the EDHF-mediated relaxation of PCA. **a**, Isolated PCA were intraluminally incubated with cationic liposomes in the absence (CTL) and presence of sense (Se), scrambled (Scr) or antisense (As) CYP 2C8/34 oligonucleotides for 3 h. Arteries were perfused for an additional 18–24 h with culture medium before isolation of endothelial cells and determination of CYP 2C expression by western blotting. Autoradiograph represents 4 additional experiments; Ponceau S staining shows equal loading of each lane. **b**, **c**, **d**, PCA treated with liposomes in the absence and presence of sense, scrambled and antisense CYP2C8 oligonucleotides were cut into rings and mounted in an organ chamber, and the relaxation to bradykinin was determined following contraction with U46619 ($0.1-1$ M). **b**, NO-mediated relaxation was assessed in the presence of diclofenac (10μ M) and sulphaphenazole (10μ M). **c**, NO- and EDHF-mediated relaxation was assessed in the presence of diclofenac (10μ M). **d**, EDHF-mediated relaxation was assessed in the presence of diclofenac and N^o-nitro-L-arginine (300μ M). Results are presented as the mean $\pm$ s.e.m. of 6 separate experiments; * $P < 0.05$ versus control.

Antisense oligonucleotides derived from the complementary DNA sequences of human CYP 2C8 and 2C9 (5'-GAGGAG-TGGGGCCAGGAGGGAG-3') were used to modulate the expression of 2C protein in PCA. This DNA sequence is located 50 base pairs (bp) downstream of the translation-initiation site within the coding region and exhibits homology only to CYP sequences within the 2C family, including the porcine 2C34. Antisense (As) oligonucleotides markedly downregulated the expression of CYP 2C in PCA endothelial cells, whereas sense (Se) and scrambled (Scr) oligonucleotides slightly increased 2C expression (Fig. 3a). Identical results were obtained using cultured porcine aortic endothelial cells.

To determine whether the downregulation of CYP 2C8/34 selectively affected EDHF-mediated relaxation, experiments were performed using isometrically contracted PCA rings. Treatment of PCA with the transfection reagent alone or with sense or scrambled oligonucleotides did not affect either the combined EDHF/nitric oxide (NO) response (Fig. 3c) or responses mediated by NO (Fig. 3b) or EDHF alone (Fig. 3d). However, EDHF-mediated relaxation, assessed in the presence of both the NOS inhibitor N^o-nitro-L-arginine and the COX inhibitor diclofenac, was selectively attenuated in rings from PCA treated with antisense oligonucleotides (Fig. 3c). Antisense oligonucleotide treatment enhanced the NO-mediated relaxation to bradykinin with or without sulphaphenazole (with diclofenac, $EC_{50} = -8.07 \pm 0.27$ M in control arteries and -8.91 ± 0.19 M in arteries treated with antisense oligonucleotides, $P < 0.01$, $n = 6$; with diclofenac and sulphaphenazole, $EC_{50} = -7.70 \pm 0.19$ M in control arteries and -9.43 ± 0.17 M in arteries treated with antisense oligonucleotides, $P < 0.001$,

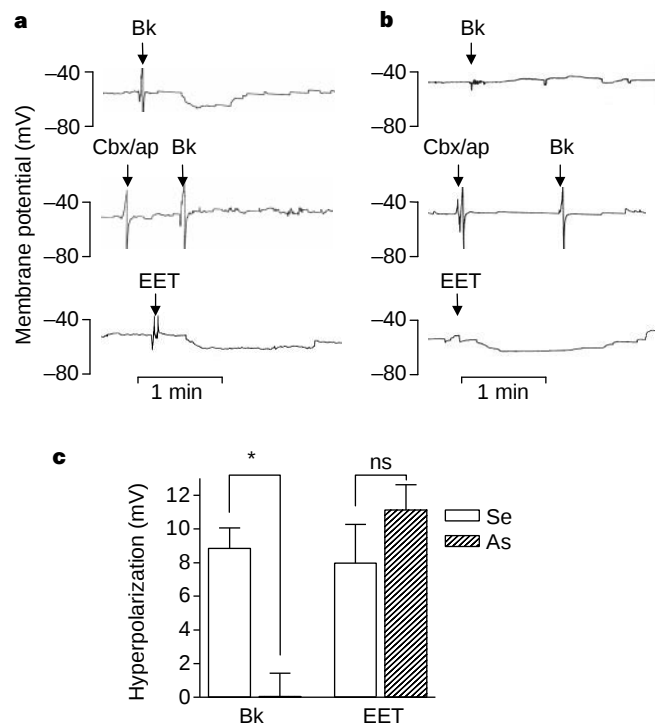


Figure 4 CYP 2C antisense oligonucleotides abolish bradykinin-induced, EDHF-mediated hyperpolarization of PCA smooth muscle cells. The effects of bradykinin (Bk; 100 nM), bradykinin with charybdotoxin (Cb; 100 nM), apamin (ap; 100 nM) and 11,12-EET (EET; 1μ M) on the membrane potential of endothelium-intact porcine coronary artery segments were determined following treatment with CYP 2C sense oligonucleotides (**a**) and CYP 2C antisense oligonucleotides (**b**). **c**, Effects of sense (Se) and antisense (As) CYP 2C oligonucleotides on the hyperpolarization induced by bradykinin and 11,12-EET. All experiments were performed in the presence of diclofenac (10μ M), N^o-nitro-L-arginine (300μ M) and U46619 (1μ M). Results are presented as the mean $\pm$ s.e.m. of 4–8 separate experiments, * $P < 0.05$ versus sense.

$n = 6$). Thus the downregulation and inhibition of CYP 2C8/34 potentiate NO-mediated relaxation, an effect that is probably due to decreased production of superoxide anions, which are a by-product of CYP activity^{20,21}.

Next we assessed the effect of CYP 2C8/34 downregulation on the bradykinin-induced hyperpolarization of PCA segments. In PCA treated with either the transfection reagent or sense oligonucleotides, bradykinin induced the hyperpolarization of smooth muscle cells (Fig. 4a). This hyperpolarization was dependent on the presence of the endothelium and was abolished by the inclusion of charybdotoxin and apamin in the perfusion solution (Fig. 4a). The CYP epoxygenase product, 11,12-EET, also elicited the hyperpolarization of PCA pretreated with sense oligonucleotides. In PCA pretreated with antisense oligonucleotides, bradykinin frequently (5 out of 8 PCA) elicited depolarization rather than hyperpolarization of vascular smooth muscle cells, whereas 11,12-EET induced hyperpolarization of similar magnitude to that observed in PCA treated with sense oligonucleotides (Fig. 4b).

Our data show that endothelial 11,12-EET production, as well as EDHF-mediated hyperpolarization and relaxation, in porcine coronary arteries can be selectively modulated by altering the expression and activity of CYP 2C8/34. This represents, to our knowledge, the first direct evidence for the existence of a CYP-dependent EDHF synthase in native endothelial cells of the coronary circulation. Moreover, as 11,12-EET was the main CYP 2C product generated by porcine coronary artery endothelial cells, this EET, which activates K_{Ca}^+ channels and hyperpolarizes vascular smooth muscle cells, is a leading candidate for the 'coronary EDHF'. □

Methods

Cultured endothelial cells

Porcine aortic and human umbilical vein endothelial cells were isolated as described⁶ and grown to confluence. Human lung microvascular and human coronary endothelial cells were purchased from Cell Systems/Clonetics. Confluent cells were then incubated with either solvent or β -naphthoflavone (3 μ M, 48 h). Thereafter protein and/or total RNA were isolated.

Preparation of PCA

Porcine epicardial artery (PCA) segments (~40 mm length; mean external diameter 2.4–2.8 mm) were excised, side branches were sealed with surgical clips and the segment was cannulated at both ends and placed into vessel chambers. After equilibration, MEM containing 10% FCS, 2.5 μ g ml⁻¹ FITC-labelled antisense, sense or scrambled oligonucleotides and 20 μ l ml⁻¹ transfection reagent (Superfect, Qiagen) were applied luminally and left in contact for 3 h under a maintained transmural pressure of 90 mm Hg. Thereafter, PCA were perfused (5 ml h⁻¹) with MEM containing 2% FCS for 18–20 h. In a separate series of experiments, PCA were perfused with MEM containing β -naphthoflavone (3 μ M, 48 h, 5 ml h⁻¹). All incubations were performed at 37 °C. Following incubation, half of each segment was cut into rings for organ chamber studies, precontracted with U46619 (0.1–1 μ M), and the relaxation to bradykinin determined in the presence of diclofenac (10 μ M) and with and without the NOS inhibitor, N^ω-nitro-L-arginine (300 μ M) as described²².

Isolation of RNA and protein from PCA

From the remaining PCA segment, we isolated endothelial cells or total RNA by intraluminal incubation with either dispase (2.4 U ml⁻¹, 45 min) or guanidine isothiocyanate. Random hexanucleotide primers were used for reverse transcription of equal amounts of RNA and the oligonucleotides used for PCR were derived from a porcine CYP 2C34 sequence (genebank accession no. U35843; upstream primer: AGACAACGAG-CACCACTCTG; downstream primer: CTTGGGGATGAGGTAGTTT) which exhibited high homology to the human 2C8 sequence (genebank accession no. Y00498). PCR products were separated on a 1.5% TAE agarose gel and visualized by staining with ethidium bromide. To verify the DNA fragment, the PCR products were transferred to nylon membranes and hybridized with a ³²P-labelled PCR product generated using a plasmid containing the coding sequence of CYP 2C8 (provided by K. A. Pritchard Jr). Phenol-soluble protein prepared from isolated PCA endothelial cells was subjected to SDS-PAGE (8%) and western blotting using two different 2C epoxygenase (rat: CYP 2C11) polyclonal antibodies (provided by E. Morgan or purchased from Daiichi Pure Chemicals).

Analysis of CYP-derived products

Native PCA endothelial cells harvested using dispase, as above, were homogenized in 200 μ l of 0.2 M KCl containing 0.01% butylated hydroxytoluene as an antioxidant and

prostaglandin B₁ (PGB₁). CYP-derived eicosanoids were extracted three times by addition of an equal volume of diethyl ether containing 0.005% BHT and 0.1% acetic acid, and the combined organic phases were evaporated under a stream of nitrogen. Dried samples were dissolved in acetonitrile/methanol (80/20, v/v) and subjected to isocratic reversed-phase (RP) HPLC with online photodiode array detection (PDAD) as described¹⁸. RP-HPLC was carried out on octadecylsilyl columns (Hypersil, Shandon; 3- μ m particles) with a mobile phase of acetonitrile/methanol/water/formic acid (54/8/38/0.001). CYP-derived metabolites were detected at 204 nm, identified by spectra plot on the peak maximum and co-elution with commercial standards, and assessed by an internal standard method using PGB₁ as standard. Analysis of full UV spectra (190–340 nm) provided by PDAD allowed accurate identification and quantification of eluting analytes by peak purity control and subtraction of possible co-eluting material.

Electrophysiological measurements

Following incubation with β -naphthoflavone or antisense, sense or scrambled oligonucleotides, PCA rings were slit and mounted in a heated chamber (37 °C) and maintained in HEPES-modified Tyrode solution containing diclofenac (10 μ M) and N^ω-nitro-L-arginine (300 μ M) and U46619 (1 μ M) to mimic conditions in the organ chamber experiments. Smooth muscle membrane potential was recorded by impaling cells through the intima. Measurements of intracellular electrical potential were made with a high impedance amplifier and glass capillary microelectrodes (tip resistance 50–150 M Ω), filled with 3 M KCl. Criteria for a successful impalement included an abrupt drop in potential on impalement, followed by a stable negative potential for at least 1 min, and a rapid return to the previous potential upon withdrawal of the microelectrode.

Statistics

Data are expressed as mean $\pm$ s.e.m. Statistical evaluation was performed using Student's *t*-test for unpaired data, one-way analysis of variance (ANOVA) followed by a Bonferroni *t*-test, or ANOVA for repeated measures where appropriate. Values of *P* < 0.05 were considered statistically significant.

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Ascaris haemoglobin is a nitric oxide-activated 'deoxygenase'

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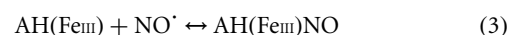
^{*} These authors contributed equally to this work.

The parasitic nematode *Ascaris lumbricoides* infects one billion people worldwide. Its peritestic fluid contains an octameric haemoglobin^{1–3} that binds oxygen nearly 25,000 times more tightly than does human haemoglobin^{4,5}. Despite numerous investigations, the biological function of this molecule has remained elusive. The distal haem pocket contains a metal, oxygen and thiol⁶, all of which are known to be reactive with nitric oxide. Here we show that *Ascaris* haemoglobin enzymatically consumes oxygen in a reaction driven by nitric oxide, thus keeping the peritestic fluid hypoxic. The mechanism of this reaction involves unprecedented chemistry of a haem group, a thiol and nitric oxide. We propose that *Ascaris* haemoglobin functions as a 'deoxygenase', using nitric oxide to detoxify oxygen. The structural and functional adaptations of *Ascaris* haemoglobin suggest that the molecular evolution of haemoglobin can be rationalized by its nitric oxide related functions.

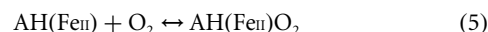
Because *Ascaris* haemoglobin has such a high affinity for oxygen, it seems unlikely that its purpose is oxygen delivery. However, there is evidence that its function might involve nitric oxide (NO). First, large amounts of oxidized haemoglobin have been found in *Ascaris* peritestic fluid⁴. This is unexpected for an oxygen-avid haemoglobin⁴. However, NO oxidizes oxygen-ligated haem groups in haemoglobin⁷, and NO synthase activity occurs in the hypodermis and muscle of *Ascaris*^{8,9}; various nitrogen oxides (NO_x) are also found in the host gut¹⁰. Second, the distal E7 residue, which is histidine in haemoglobins from most higher organisms, is a glutamine in *Ascaris* haemoglobin. Substitution of the E7 histidine

with glutamine increases ~1,000-fold the reactivity of oxidized myoglobin with NO¹¹. Third, the crystal structure of an *Ascaris* haem domain⁶ reveals a cysteine in close proximity to the ligand-binding site. NO is transferred between haem and thiol in human haemoglobin^{7,12–14}, and we find that fresh *Ascaris* peritestic fluid contains endogenous S-nitrosothiol (SNO). The presence of these components of NO biology indicate that NO may be involved in the function of *Ascaris* haemoglobin.

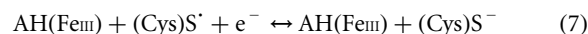
We constructed optical spectra for various liganded, unliganded and oxidized forms of *Ascaris* haemoglobin (AH), from which difference spectra were derived (not shown). Absorption-difference spectroscopy was used to examine the reaction of NO with the haemoglobin. NO was titrated against 6 μM haemoglobin (haem content) in 1.8-μM steps. Addition of NO resulted in the immediate oxidation of haemoglobin to methaemoglobin (Fig. 1a), whereas oxidation of haemoglobin by ferricyanide⁴, which requires dissociation of liganded oxygen, requires several minutes. The peak ferric (Fe^{III}) haem yield was seen after the addition of 19.8 μM NO (three NO molecules per haem) (Fig. 1a). Addition of further NO, to a total of 45 μM, induced the accumulation of AH (Fe^{III}) NO. These data indicate that NO is directly oxidizing AH(Fe^{II})O₂ to methaemoglobin (reactions 1 and 2 below); that NO reacts with methaemoglobin to form AH(Fe^{III})NO (reaction 3); and that additional reactions must be occurring.



We used photolysis-chemiluminescence to measure the NO content of 6 μM haemoglobin (haem content) following the step-wise addition of 45 μM NO, as described above. The haemoglobin contained 7.8 μM NO, of which 5.1 μM (65%) was SNO (results not shown), consistent with an equilibrium that is known to occur between Fe^{III}NO in mammalian haemoglobin and SNO (reaction 4). However, the transfer of NO⁺ from haem to thiol would inevitably be coupled to the binding of oxygen (reaction 5), which was not detected. Moreover, most of the added NO did not form either a chemiluminescence-detectable or spectrally active nitrosyl species.



Accordingly, we hypothesized that NO might be consumed in a reaction with oxygen to form nitrate (reaction 6), but this reaction would require a source of electrons (reaction 7).



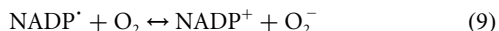
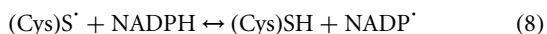
Previous work has shown that *Ascaris* haemoglobin can reduce oxidized cytochrome *c* in an NADPH-dependent manner¹⁵. NADPH was found to increase the efficiency with which haemoglobin was oxidized by NO. Moreover, very little AH(Fe^{III})NO

Table 1 Endproduct analysis of NO metabolism by *Ascaris* haemoglobin

Concentration of DEANO added (μM)	NO ₂ ⁻ (μM), no haemoglobin	NO ₃ ⁻ (μM), no haemoglobin	NO ₂ ⁻ (μM), with haemoglobin	NO ₃ ⁻ (μM), with haemoglobin
~2	0.0 ± 0.09	0.0 ± 0.7	0.0 ± 0.27	1.2 ± 0.11
~4	0.8 ± 0.04	1.0 ± 0.12	0.1 ± 0.17	8.5 ± 0.12
~8	5.7 ± 0.52	8.1 ± 0.27	2.4 ± 0.07	11.6 ± 0.29

Varying amounts of DEANO were added to solutions containing 500 μM NADPH with or without *Ascaris* haemoglobin (1.5 μM haem content). Yields of nitrite and nitrate were determined by the Greiss reaction and high-performance capillary electrophoresis. Values are mean ± standard error for three experiments.

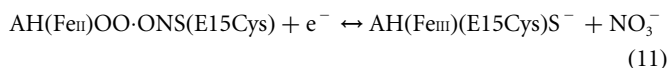
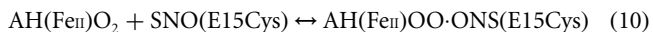
(1.6 μM) and no AH-SNO were detected in the presence of NADPH, even after adding 7.5 NO per haem (Fig. 1b), indicating that the haemoglobin metabolizes NO in an NADPH-dependent manner. The thiyl radical produced in reaction 6 could be reduced by NADPH (reaction 8), ultimately generating O_2^- (reaction 9). The NO complexes that build up in the absence of NADPH may be considered as reaction intermediates.



Incubation of haemoglobin with S-nitrosocysteine, under conditions that selectively S-nitrosylate human haemoglobin¹³, produced AH-SNO (Fig. 1c). In addition, the haems were oxidized and much haem-bound NO was detected. As might be predicted from reactions 4–6, adding NADPH decreased the amounts of haem-bound NO and SNO (Fig. 1c), and nitrate (NO_3^-) accumulated in the reaction mixtures. Coupling of haem and thiol was further indicated by studies of a mutant haem domain (D1), in which B10 tyrosine was substituted with leucine. Treatment with S-nitrosocysteine resulted in the formation of D1-SNO and the reduction of the already oxidized haems (results not shown). These data indicate that the thiol(s) and haem(s) are redox partners that transfer NO and/or electrons, and that NO is metabolized in these reactions.

Each polypeptide in *Ascaris* haemoglobin contains two tandem globin domains. The first, D1, has three cysteine residues (A7, E15 and E19) that are conserved in the second globin fold, D2 (refs 1, 3, 4). To determine their function, we examined the effects of S-nitrosylation of recombinant D1 and mutants in each of the three cysteines (Fig. 1d). Treatment with S-nitrosocysteine induced

oxidation of native D1 and all of the mutants. Mutation of the E19 cysteine, located in the proximal haem pocket (analogous to human Cys β 93), had only a small effect on the formation of both S-nitrosothiol and haem-bound NO. In contrast, mutation of the E15 cysteine, which lies near the ligand-binding site⁶, blocked SNO formation and production of haem-bound NO. The crystal structure of D1 indicates that the distal pocket may facilitate interactions between the dioxygen bound to haem and the NO bound to E15 thiol, forming a peroxynitrosyl intermediate that rearranges to nitrate (reactions 10, 11). An NO/oxygen intermediate involving Fe and thiol has been identified in the crystal structure of nitrile hydratase¹⁶.



Mutation of the surface A7 thiol also prevented the S-nitrosylation of E15 by S-nitrosocysteine. These observations point to initial transnitrosation of the surface A7 cysteine, followed by intramolecular transfer of the NO group to internal thiols, most notably E15 cysteine, and then to haem. This route may channel NO groups deriving from endogenous SNOs to the haemoglobin active site, whereas free NO gains access via the haem.

NO was added to haemoglobin and its concentration directly monitored using an electrochemical probe. The haemoglobin consumed NO compared with haemoglobin-free solutions (Fig. 2a) at a rate approximately equivalent to the concentration of haem present (1.5 μM haem, 1.9–2.1 μM NO consumed), indicating a single direct reaction of NO with $\text{AH}(\text{Fe}^{\text{II}})\text{O}_2$. In these experiments without NADPH, the rate of NO decay matched that observed in buffer.

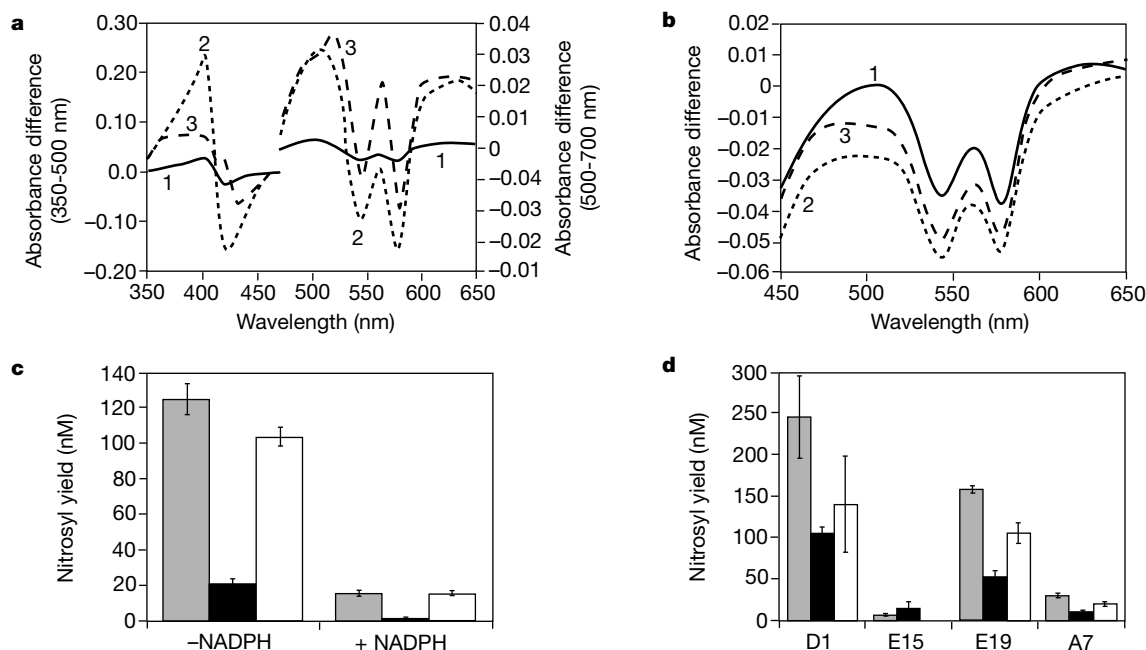


Figure 1 Interaction of native *Ascaris* haemoglobin (AH) with NO. **a**, NO titration of haemoglobin in the absence of NADPH. Repeated additions of aqueous NO (1.8 μM) were made to haemoglobin (6 μM haem content). Spectra were recorded immediately after mixing. Results are shown as difference spectra against $\text{AH}(\text{Fe}^{\text{II}})\text{O}_2$. Addition of NO led to the rapid appearance of a little $\text{AH}(\text{Fe}^{\text{II}})$ (spectrum 1, solid line), as seen by an increase in absorbance at ~ 400 nm. A peak $\text{AH}(\text{Fe}^{\text{II}})$ difference spectrum was observed after 11 additions of NO (19.8 μM) (spectrum 2, dotted line). Subsequent additions of NO up to 45 μM led to the build-up of $\text{AH}(\text{Fe}^{\text{II}})\text{NO}$ (spectrum 3, dashed line), detected by small peaks at 418 and 519 nm and an increase at 563 nm. **b**, NO titration of haemoglobin in the presence of 500 μM NADPH. Addition of ~ 2 μM NO led to $\text{AH}(\text{Fe}^{\text{II}})$ production (spectrum 1). The peak $\text{AH}(\text{Fe}^{\text{II}})$ yield was produced by 14.4 μM NO (spectrum 2).

Addition of up to 45 μM NO (spectrum 3, $\text{AH}(\text{Fe}^{\text{II}})$) does not produce $\text{AH}(\text{Fe}^{\text{II}})\text{NO}$.

c, S-nitrosylation of native haemoglobin. Haemoglobin (178 μM haem) was incubated with S-nitrosocysteine (twofold molar excess over haem) in the absence (–) or presence (+) of NADPH under conditions favouring selective transnitrosation of thiols¹³. Photolysis–chemiluminescence was used to measure the total nitrosyl content of haemoglobin (grey bars), $\text{AH}(\text{Fe}^{\text{II}})\text{NO}$ (black bars) and SNO (white bars). S-nitrosocysteine rapidly oxidized haem in both the presence and absence of NADPH (not shown). **d**, Identification of NO-reactive cysteines. S-nitrosylation of recombinant D1 and D1 mutants with serines substituted for cysteines (A7, E15 and E19) was performed with a 10-fold molar excess of S-nitrosocysteine. Results were standardized to 113 μM haem; total nitrosyl (grey bars), FeNO (black bars) and SNO (white bars) contents are shown.

In contrast, NO decay by haemoglobin was accelerated more than 10-fold in the presence of NADPH (Fig. 2a). Thus, AH enzymatically metabolizes NO in an NADPH-dependent manner.

We used stopped-flow spectrophotometry to gain further insight into the mechanism of NO consumption. Adding 50 μM NO to 6 μM haemoglobin in the absence of NADPH (Fig. 2b) resulted in the rapid formation of AH(FeIII)NO, followed by a build-up of AH(FeIII)NO. AH(FeIII)NO was formed before complete oxidation of AH(FeII)O₂. Adding 500 μM NADPH (Fig. 2c) slowed the initial rate of NO-induced conversion of AH(FeII)O₂ to AH(FeIII) roughly threefold, but increased the net yield of oxidized haemoglobin and prevented detectable AH(FeIII)NO from forming. The slower build-up of AH(FeIII) in the presence of NADPH, and the early detection of AH(FeIII)NO in its absence, indicates that AH(FeIII) competes with AH(FeII)O₂ for NO. The reduced rate of AH(FeIII) formation in the presence of NADPH is probably a result of NO turnover.

The products of NO consumption by *Ascaris* haemoglobin were determined by using the NO donor diethylamine NONOate (DEANO), 5 μM of which released NO over 2 min (Fig. 2d). When haemoglobin was present, however, NO was undetectable; that is, haemoglobin consumed NO. Varying amounts of DEANO (2–8 μM , yielding 4–16 μM NO) were incubated in solutions of NADPH with or without *Ascaris* haemoglobin (1.5 μM haem). In the absence of haemoglobin, 2 μM DEANO produced no detectable nitrite or nitrate (Table 1), presumably owing to loss of NO to the atmosphere. However, in the presence of haemoglobin, NO was effectively captured and fixed as nitrate. Furthermore, haemoglobin metabolized larger concentrations of NO, primarily to nitrate, both

at high and low oxygen pressure ($p\text{O}_2$) whereas, in the absence of haemoglobin, nearly equimolar levels of nitrite and nitrate were observed. Taken together, these data indicate an enzymatic function for haemoglobin in reacting NO with oxygen to produce nitrate. We have previously identified NO oxygenase activity with the bacterial flavohaemoglobin HMP¹⁷. By comparison, HMP is by far the more efficient at metabolizing NO.

We then examined the consumption of oxygen by haemoglobin using a Clark electrode in a sealed vessel (Fig. 3a). Incubation of purified haemoglobin with NADPH caused a slow decrease in $p\text{O}_2$ in the absence of NO. The visible spectrum of oxygen-liganded haemoglobin did not change with falling $p\text{O}_2$, in keeping with its extraordinarily high affinity for oxygen. In fully deoxygenated buffer, NADPH converted the protein to the deoxy form (Fig. 3b). *Ascaris* haemoglobin therefore exhibits intrinsic NADPH oxidase activity (for example, reactions 8, 9).

Addition of NO increased the rate of oxygen consumption; indeed, ~43 μM oxygen was consumed by the addition of only 10 μM NO (Fig. 3a). Even taking into account a background rate of oxygen consumption, *Ascaris* haemoglobin transformed at least two O₂ molecules per NO ($n = 7$ experiments). These results indicate that there are additional reduction pathways for oxygen, beyond fixation as nitrate, which are primed by NO. A peroxidase or oxidase reaction is a reasonable possibility, as haemoglobins carry out peroxidase and oxidase functions that may be catalysed by NO-related species¹⁸. Moreover, redox cofactors, such as the E15 thiol within *Ascaris* haemoglobin, support this chemistry in other haemoglobins¹⁹ (reactions 12, 13). Alternatively, oxygen consumption

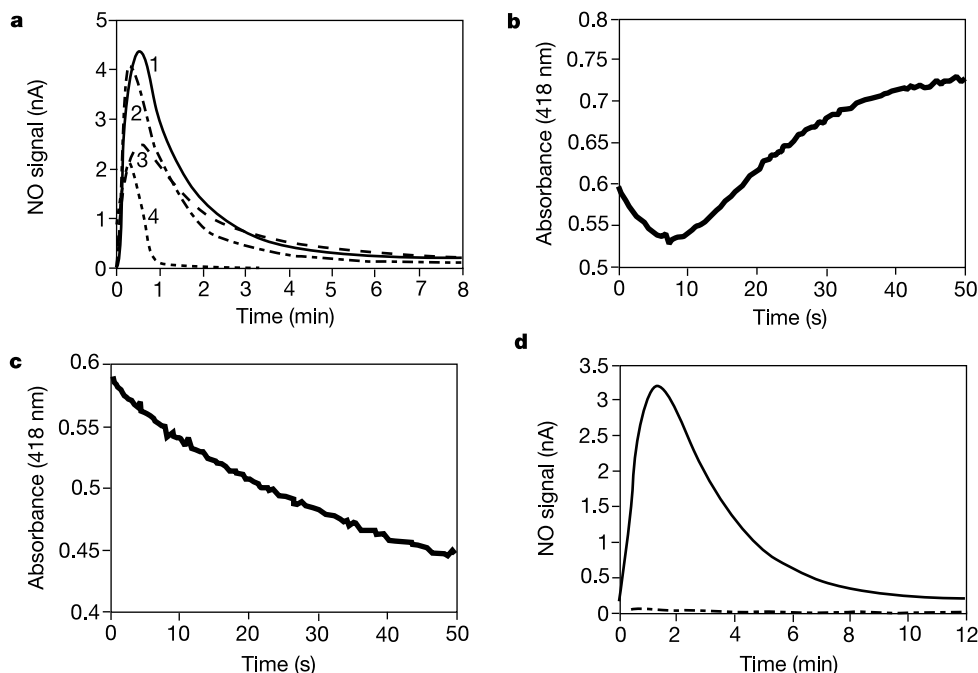
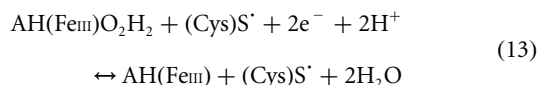
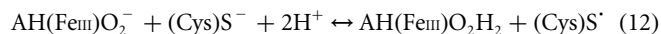


Figure 2 Consumption of NO by *Ascaris* haemoglobin. **a**, A NO electrode was used to measure the consumption of 6 μM NO. Adding NO to buffer (PBS, pH 6) (solid line, 1) resulted in a peak height of 4.4 nA that slowly decayed. Adding NO to buffer containing 500 μM NADPH (short-long dashed line, 2) yielded a similar NO signal (4.2 nA peak height) that decayed at a similar rate. Adding NO to haemoglobin (1.5 μM haem) without NADPH (dashed line, 3) produced a peak signal of only 2.5 nA, consistent with stoichiometric reaction of NO with haem-bound oxygen, but decay was similar to NO alone. Adding NO to haemoglobin (1.5 μM haem) plus 500 μM NADPH (dotted line, 4) further decreased the NO signal (2.0 nA) and increased the rate of decay (decay in 1 min against >10 min without haemoglobin or NADPH). **b**, Kinetics of haemoglobin interaction with NO in the absence of NADPH. AH(FeII)O₂ (6 μM haem) was mixed with (25 μM) DEANO in a stopped-flow spectrophotometer. Before mixing, all solutions were

deoxygenated by bubbling with argon gas for 45 min. Spectral changes at 418 nm (AH(FeII)O₂) are shown. When NO was added, absorbance at 418 nm rapidly decreased (within 8 s) from 0.6 to 0.53 (~47% loss of AH(FeII)O₂) as the AH(FeIII) intermediate formed. The rise in absorbance is due to the build-up of AH(FeIII)NO. Thus AH(FeIII) competes with AH(FeII)O₂ for NO. **c**, Interaction of haemoglobin with NO in the presence of NADPH. AH(FeII)O₂ (6 μM haem) was mixed with (25 μM) DEANO plus 500 μM NADPH (final) under low $p\text{O}_2$. When NO is added, absorbance at 418 nm decreases over 50 s from 0.6 to 0.45, and build-up of AH(FeIII)NO is not seen. **d**, Haemoglobin consumption of NO released by DEANO. DEANO (5 μM , final) was added to a solution containing 500 μM NADPH (solid line) (peak, 3.2 nA). Adding (5 μM) DEANO with haemoglobin (1.5 μM haem) and 500 μM NADPH did not generate a detectable signal (dashed line).

may be attributed to a cytochrome P450-like activity of haemoglobin.



We have used these data to construct a model for the consumption of O_2 and NO. The distal pocket of haemoglobin contains a strong hydrogen-bonding network between liganded oxygen, B10 tyrosine and E7 glutamine^{6,20}. The hydrogen-bonded oxygen has a superoxide character^{21–23} (reaction 1). NO entry into the distal pocket therefore produces haem oxidation and nitrate⁷ (reaction 2). Once methaemoglobin is generated, it efficiently binds NO (reaction 3); the presence of a distal glutamine speeds the reaction¹¹. This $\text{AH(Fe}^{\text{III}}\text{)NO}$ intermediate is in equilibrium with SNO(E15Cys) (reaction 4), a conclusion supported by photolysis–chemiluminescence, stopped-flow analyses and the ability of S-nitrosothiol to oxidize haems in *Ascaris* but not mammalian^{13,14} haemoglobin. Oxygen then binds to the ferrous haem of SNO -containing molecules (reaction 5). We suggest that this generates an unstable peroxynitrosyl complex within the distal pocket, which decomposes to produce nitrate (reactions 6–11). Involvement of

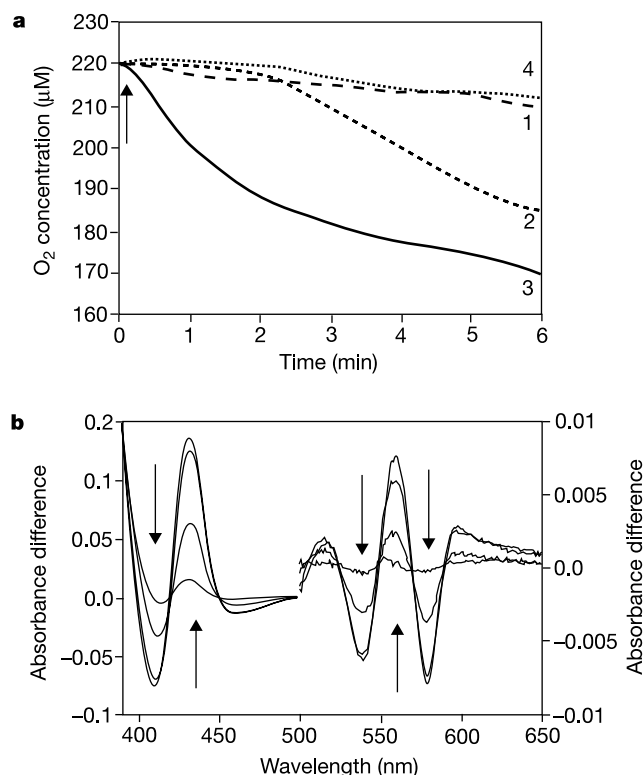


Figure 3 Oxygen consumption by *Ascaris* haemoglobin. **a**, A Clark electrode was placed in a sealed glass vessel. Adding ($7.5 \mu\text{M}$) NO produced little reduction in $p\text{O}_2$ (dashed line, 1). Adding $500 \mu\text{M}$ NADPH to buffer containing haemoglobin ($0.43 \mu\text{M}$) resulted in oxygen consumption after a 2.5-min lag phase, suggesting an autocatalytic process (dotted line, 2). NO ($7.5 \mu\text{M}$) added to buffer containing haemoglobin and NADPH led to rapid consumption of oxygen (solid line, 3) whereas no oxygen was consumed without NADPH (close dots, 4) or if NADPH was added 2.5 min later (not shown). Arrow indicates NO addition in lines 1, 3 and 4, and NADPH addition for line 2. Data are representative of three similar experiments. **b**, PBS (pH 6.0) containing $3 \mu\text{M}$ haemoglobin was deoxygenated by bubbling with argon for 45 min. After taking the spectrum, $500 \mu\text{M}$ NADPH was added and the reaction was followed for 10 min (arrows). Spectra are the difference versus the pre-NADPH addition spectrum, and show a progression from Fe(II)O_2 to an unliganded Fe(II) state.

(thiyl) radical chemistry (reaction 6) is consistent with a peroxidase function^{18,19,24} (reactions 12, 13). In particular, thiyl radical-induced peroxide (Fe(III) haem complex) has been shown to be generated in mammalian haemoglobin¹⁹. We do not know which NO-related species primes *Ascaris* haemoglobin for O_2 metabolism, but the peroxynitrosyl intermediate is an excellent catalytic candidate. By serving as a substrate for such haemoproteins, peroxynitrite is known to activate peroxidase activity¹⁸. These mechanistic issues notwithstanding, our data clearly demonstrate that *Ascaris* haemoglobin consumes NO and oxygen in an NADPH-dependent manner.

Ascaris suum adults metabolize anaerobically, and it is thought that free oxygen is highly toxic to them.²⁵ To establish the physiological importance of haemoglobin-induced deoxygenation, we measured $p\text{O}_2$ in the intestines of pigs, where *A. suum* is found, and both the $p\text{O}_2$ and NO content of the perienteric fluid of worms. *A. suum* can migrate throughout the gut but tends to reside in the

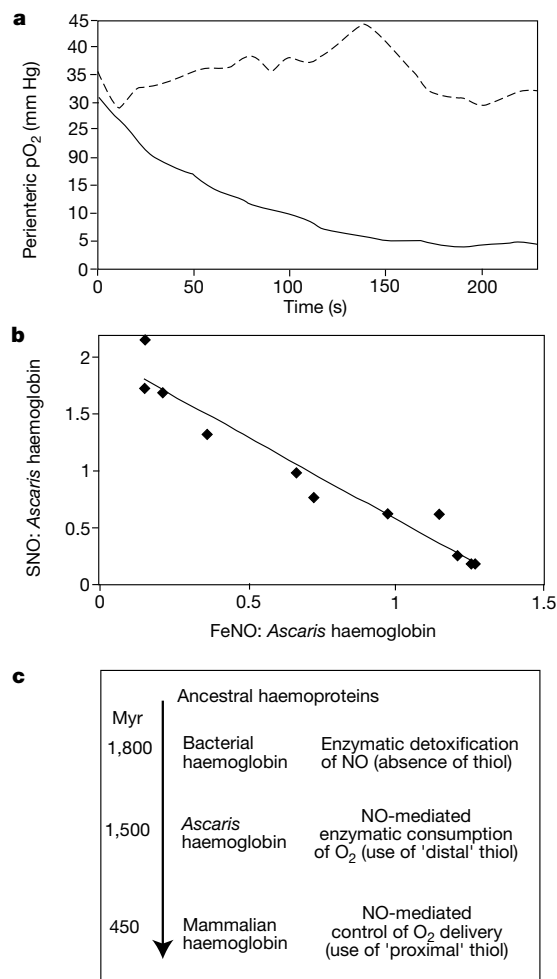


Figure 4 Analyses of fresh *Ascaris* worms and related evolutionary implications.

a, Perienteric fluid has a low $p\text{O}_2$. A cannula was inserted into the perienteric space ~ 1 cm below the head, through which a fibre-optic O_2 probe was passed. Probe output ($p\text{O}_2$) drops (with passage into the cavity) to 4 mm Hg (solid line). A second cannula drained the perienteric fluid, after which perienteric $p\text{O}_2$ rises to 40 mm Hg (dashed line).

b, Perienteric fluid contains SNO and FeNO. The perienteric fluid of fresh adult female *Ascaris* worms (data points) was collected and analysed by photolysis–chemiluminescence for SNO and FeNO¹³. Data were normalized to *Ascaris* haemoglobin content.

c, Evolution of haemoglobin rationalized by its NO-related functions. Nematode haemoglobin is at the divide between primordial and mammalian haemoglobins²⁶ and highlights the transformation of a NO detoxification function into a respiratory and NO delivery function. Distal and proximal refer to the position of cysteine with respect to the haem pocket. Myr, millions of years ago.

jejunum. We made 40 measurements from the jejunum in two pigs and found a significant amount of O_2 , with an apparent pO_2 gradient from the intestinal wall (~ 10 mm Hg) to the lumen (~ 0 mm Hg). We collected 11 worms from pig intestines. The perienteric cavity was cannulated in three worms and a fibre optic O_2 probe was inserted. Even in room air, the worms consistently maintained a cavity pO_2 of 4 mm Hg (Fig. 4a). Drainage of haemoglobin-containing perienteric fluid from the cavity markedly increased the tissue pO_2 to ~ 40 mm Hg (Fig. 4a). In freshly collected perienteric fluid from individual worms we found $6.15 \pm 0.37 \mu\text{M}$ bound NO ($n = 11$) (1–2 NO molecules per octamer haemoglobin), which was present as SNO and metal nitrosyl (Fig. 4b). Moreover, the amount of SNO in perienteric fluid was inversely correlated with metal nitrosyl content, consistent with functional coupling between haem and thiol (reaction 4). These data support the idea that *Ascaris* haemoglobin is a nitric oxide-dependent 'deoxygenase', using endogenously produced NO substrate to detoxify oxygen.

In the phylogeny of haemoglobins²⁶, that of nematodes sits at the divide between the primordial bacterial flavohaemoglobins, which have been shown to function in NO/SNO detoxification^{17,27,28}, and the cooperative mammalian haemoglobins, which function in NO/SNO delivery^{12–14}. An NAD(P)H-dependent oxido-reductase activity supports NO metabolism in bacteria, whereas critical thiols subserve the NO donor function that regulates O_2 delivery in mammals. *Ascaris* haemoglobin seems to represent an 'evolutionary bridge': it retains a primitive enzymatic function, albeit one that is adapted to control oxygen concentration. Moreover, it controls pO_2 with NO^{12–14,14}. The positioning of a thiol in the distal pocket of nematode haemoglobin, as opposed to the proximal pocket in mammalian haemoglobin (R structure), enables the alternative NO-related functions of deoxygenation and oxygenation, respectively. The primordial NO-detoxifying function of haemoglobin has therefore been transformed into a respiratory function by incorporating thiols that allow haemoglobin to use, rather than simply metabolize, NO (Fig. 4c). Although the primary function of *Ascaris* haemoglobin seems to be oxygen removal, it may also protect against NO normally present in the host gut¹⁰ or generated by innate host defences, rather like the bacterial flavohaemoglobin that metabolizes NO^{17,27,28}, albeit more efficiently and by an entirely different mechanism.

We have discovered new NO chemistry involving enzymatic activity of a haem-thiol-NO redox triad, and have shown that *Ascaris* haemoglobin functions to detoxify oxygen. The unique structural adaptation of *Ascaris* haemoglobin, nearly 1,500 million years ago, establishes a new paradigm in which haemoglobins have evolved for distinct NO-related functions. □

Methods

Mutant haemoglobin constructs

Cloning and characterization of *Ascaris* haemoglobin domain one (D1) and mutant D1 B10YL (in which B10 tyrosine was substituted with leucine) have been described²⁹. D1 (A7 cysteine $\rightarrow$ serine) was generated by the polymerase chain reaction (PCR) using D1 complementary DNA as a template with a forward primer containing the mutation (5'-GCATCCATGGCGAATAAAACGAGAGAACTATCCATGAAATCACTCGAA-3') and a reverse primer to the extreme 3' end of the gene²⁹. PCR strategy was used to mutate each of the other two D1 cysteines to serines. D1 cDNA was used as a template and the forward and reverse primers were to the extreme 5' and 3' ends of D1. Mutant primers were as follows: D1 with E15 cysteine (residue 72) mutated to serine (E15CS) 5'-CTCTTGGCAA GCCACGTTCTT-3' and its complement; and D1 with E19 cysteine (residue 76) mutated to serine (E19CS) 5'-GCATGCCACGTTCTTCCGCCACCTACGATGAC-3' and its complement. Mutant D1 constructs were cloned into pET-8C.

Haemoglobin purification

Native *Ascaris* haemoglobin was pelleted from the haemolymph of freshly obtained *A. suum* (Carolina Biological Supply Co.) by ultracentrifugation at 80,000g for 16 h. Haemoglobin was further purified by fractionation on a Waters DEAE-5PW anion-exchange column eluted with a linear gradient of 50–500 mM NaCl. Purified globins were >95% of all protein, as assessed by SDS-PAGE (data not shown).

Preparation of deoxy, ferrous-nitrosyl, ferric and ferric-nitrosyl haemoglobin

Deoxy native *Ascaris* haemoglobin was obtained by >10 min incubation of AH(FeII) O_2 with sodium dithionite. AH(FeII)NO was produced by adding excess NO. AH(FeII) O_2 (6 μM haem) was incubated overnight with 50 μM potassium ferricyanide to oxidize haems. To obtain AH(FeIII)NO, 18 μM NO (final concentration) was added to ferricyanide-oxidized haemoglobin. Spectra were recorded in a Perkin Elmer UV/Vis Spectrometer, Lambda 2S. Haem content was assessed by the pyridine haemochromagen method.

Titration of *Ascaris* haemoglobin with NO

NO saturated solutions were 1.2–1.8 mM¹². Nitric oxide stock solution was added sequentially from a gas-tight Hamilton syringe with a Teflon seal to 1 ml haemoglobin (6 μM haem content) in phosphate-buffered saline (PBS), pH 6, with or without 500 μM NADPH. Spectra were immediately recorded after each addition of NO.

NO consumption

A Clark-type NO electrode (Iso-NO, World Precision Instruments) immersed in a stirred glass vial was used. NO was added at a final concentration of 6 μM to haemoglobin (1.5 μM haem content) in PBS, pH 6, with or without 500 μM NADPH, or the NO donor DEANO was added. Samples were assayed for nitrite and nitrate by the Greiss reaction and high-performance capillary electrophoresis (Applied Biosystems)¹⁷.

S-nitrosylation of haemoglobins

Transnitrosation of globins was as described¹³. Globins were incubated with a 2- to 10-fold excess of S-nitrosocysteine in 10% v/v Borax, 100 μM diethylenetriaminepentaacetic acid (DTPA), pH 9. SNO and Fe-NO were measured by photolysis–chemiluminescence with a sixfold molar excess of $HgCl_2$ (ref. 12).

Kinetic analysis of haemoglobin

An Applied Photosystems stopped-flow spectrophotometer was used. Study conditions: haemoglobin, 6 μM haem content; DEANO, 25 μM ; NADPH, 500 μM . Solutions were deoxygenated by bubbling with argon for 45 min. Data were analysed by using Pro-K software for the SX.18MV.

Oxygen consumption

PBS (2 ml, pH 6) was placed in a sealed glass vessel with a Clark electrode. NADPH, NO or haemoglobin was added through a capillary opening with a gas-tight Hamilton syringe. Data were collected with an analogue chart recorder.

Fibre-optic analysis of oxygen content

pO_2 in the jejunum of the pig and in the *Ascaris* perienteric space was measured using OxyLite (Oxford Optronix)³⁰. The system consists of a fibre-optic probe with an immobilized oxygen-sensitive dye, ruthenium chloride, at the tip. When this dye is excited by pulsed laser light, the timecourse of the decay is a function of local pO_2 .

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A chain initiation factor common to both modular and aromatic polyketide synthases

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Antibiotic-producing polyketide synthases (PKSs) are enzymes responsible for the biosynthesis in *Streptomyces* and related filamentous bacteria of a remarkably broad range of bioactive metabolites, including antitumour aromatic compounds such as mithramycin¹ and macrolide antibiotics such as erythromycin². The molecular basis for the selection of the starter unit on aromatic PKSs is unknown³. Here we show that a component of aromatic PKS, previously named 'chain-length factor'⁴, is a factor required for polyketide chain initiation and that this factor has decarboxylase activity towards malonyl-ACP (acyl carrier protein). We have re-examined the mechanism of initiation on modular PKSs and have identified as a specific initiation factor a domain of previously unknown function named KSQ, which

operates like chain-length factor. Both KSQ and chain-length factor are similar to the ketosynthase domains that catalyse polyketide chain extension in modular multifunctional PKSs and in aromatic PKSs, respectively, except that the ketosynthase domain active-site cysteine residue is replaced by a highly conserved glutamine in KSQ and in chain-length factor. The glutamine residue is important both for decarboxylase activity and for polyketide synthesis.

Aromatic PKSs resemble typical bacterial fatty-acid synthases (FASs) in containing a few largely monofunctional enzyme subunits (type II organization). Each aromatic PKS contains a set of three essential subunits (the ketosynthase (KS), the chain-length factor (CLF)⁴ and the acyl carrier protein (ACP)), which is referred to as the minimal PKS⁵. In addition, aromatic PKSs typically contain other enzyme subunits, including specific ketoreductases, cyclases and aromatases which interact with the growing poly-β-ketoacyl-ACP intermediate to produce characteristic polycyclic aromatic products. Heterologous expression of genes from different aromatic PKS gene clusters has led to the *in vivo* production of many novel polyketide products³, and to the formulation of 'design rules' for the rational production of such products by the appropriate mixing and matching of subunits⁶. These design rules have since required modification, particularly with regard to the proposal, originally based on very limited experimental evidence, that CLF determines the chain length. It is now accepted that a heterologous CLF may not alter the product chain length as predicted^{7,8}, that CLF exerts major influence only in the presence of its cognate KS, and that ketoreductases, cyclases^{7–9} and aromatases¹⁰ may all have a decisive influence on the outcome.

Recent studies on the expression of components of bacterial aromatic PKSs *in vitro* have shown that the purified minimal PKS, namely KS/CLF and ACP, can produce the expected polyketide products when incubated with malonyl-CoA^{11–13}. There is disagreement^{12,13} over whether polyketide synthesis *in vitro* absolutely requires the presence of purified FAS malonyl-CoA:ACP acyl transferase (MCAT)^{11,12}, as opposed to self-malonylation by the PKS ACP¹⁴; however, it is agreed that malonyl-CoA and not acetyl-CoA is the precursor of starter units on the actinorhodin PKS^{11,14} and the tetracenomycin PKS¹². This required decarboxylation has been assumed^{11,12} to be a function of the KS active site, by analogy with similar decarboxylation that is known to occur on the FAS¹⁵ (Fig. 1), and that has been proposed to occur in individual KS active sites of the modular erythromycin PKS¹⁶. The KS and CLF subunits of an aromatic PKS are similar in sequence¹⁷, but the KS active site has an essential^{18,19} conserved cysteine (C), whereas in CLF the corresponding residue is a highly conserved glutamine (Q) (Fig. 2). This is striking because when the essential²⁰ active-site cysteine of the animal FAS is treated with iodoacetamide, it is specifically converted

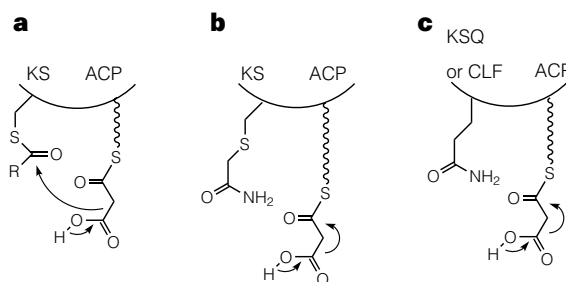


Figure 1 Decarboxylation of malonyl-ACP on KS, KSQ and CLF domains. **a**, A typical decarboxylative chain-elongation step in FASs and PKSs. **b**, Decarboxylation of malonyl-ACP by FAS¹⁵ upon modification of the active-site cysteine with iodoacetamide. **c**, Proposed common mechanism of formation of enzyme-bound starter units in type I PKSs containing an amino-terminal KSQ domain and in type II PKSs by the CLF subunit. The conserved glutamine in KSQ and CLF is proposed to have a direct role in catalysis.

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into a carboxamidomethylcysteine residue that is very similar in size, shape and polarity to glutamine¹⁵ (Fig. 1). The treated enzyme is thereby converted from a ketosynthase into a malonyl-CoA decarboxylase¹⁵ and all fatty acid synthesis is abolished. In the normal course of the KS reaction, C–C bond formation is presumably promoted by the presence of an acyl unit on the cysteine. The presence of glutamine instead of cysteine in CLF might render it an effective decarboxylase, and therefore CLF would have the properties required of a chain initiation factor that generates acetyl-ACP *in situ* on the minimal PKS.

We tested this idea using the 'minimal' actinorhodin (*act*) PKS, consisting of KS, CLF and ACP components, which together synthesize the octaketides 1 and 2 *in vitro*³ (Fig. 3). A number of site-specific mutants of the KS (Cys 169) and CLF (Gln 161) subunits of the *act* PKS were constructed. First, mutants were made in which the KS cysteine was substituted by alanine. Such mutants are clearly incapable of catalysing polyketide synthesis. These mutant KS/CLF complexes were purified to homogeneity, and tested for their ability to decarboxylate malonyl-ACP, monitoring the appearance of acetyl-ACP by electrospray mass spectrometry (ESMS)¹⁴. Figure 4a shows that for the mutant KS(Ala)/CLF(Gln) approximately 40% decarboxylation of malonyl-ACP to acetyl-ACP occurred in 60 min under the conditions used (relative molecular mass (M_r) for malonyl-ACP: found, 9,527.6; calculated, 9,528; M_r for acetyl-ACP: found, 9,483.9; calculated, 9,484). A time course for decarboxylation by this mutant is shown in Fig. 4b. Malonyl-ACP was recovered unchanged from the control incubation (no added enzyme) after 60 min (data not shown). The mutant KS(Ala)/CLF(Ala) had no decarboxylase activity and the malonyl-ACP was again recovered unchanged (data not shown). These findings unequivocally implicate the CLF and its active-site glutamine in the provision of the starter unit for aromatic polyketide production. Unexpectedly, the mutant KS(Gln)/CLF(Ala), which was designed to mimic the chemical modification of the KS active-site cysteine with iodoacetamide¹⁵, almost completely decarboxylated malonyl-ACP within one minute under these conditions, as judged by ESMS (Fig. 4a). The single amino-acid change from cysteine to glutamine seems sufficient to convert one activity into the other; however, further work is needed to understand why this mutant is such a potent decarboxylase. The mutant KS(Cys)/CLF(Ala) was also tested for its ability to decarboxylate malonyl-ACP and to synthesize the predicted polyketide products SEK4 (octaketide 1) and SEK4b (octaketide 2) (Fig. 3)¹¹. A significant amount of decarboxylation (up to 20% after 60 min) was seen (data not shown), implying that the KS is also competent to decarboxylate malonyl-ACP and that malonyl-ACP decarboxylase activity of CLF is not absolutely essen-

tial for *in vivo* production of polyketides. The role of CLF in the provision of starter units was further examined by monitoring the production of octaketides 1 and 2 by this mutant (Fig. 4c). In the absence of added acetyl-ACP, polyketide synthesis proceeded only after an evident lag. As expected, the rate of production of polyketides was increased and the lag phase abolished by the presence of acetyl-ACP (Fig. 4c), showing that decarboxylation of malonyl-ACP by the KS alone is not sufficient to sustain maximal levels of polyketides synthesis.

The type I PKSs for macrolide antibiotics are giant multifunctional enzymes containing different sets or modules of FAS-related enzyme activities for each round of polyketide chain extension². The extensively studied erythromycin PKS (6-deoxyerythronolide B synthase (DEBS)) has a discrete loading module containing a propionyl-CoA:ACP acyltransferase (AT) and an ACP domain². For the erythromycin PKS, which is typical of those that do not have a KSQ domain in their loading module, evidence indicates that non-carboxylated acyl-CoA species are the substrates for the loading module^{21,22}; however, recent DNA sequencing efforts have shown that it is more typical for a modular PKS to have a loading module containing an additional KS-like domain (Fig. 2). This domain contains a glutamine in place of the essential cysteine²³ at the active site (Fig. 2) and has no known function. To examine whether it functions as an initiation factor analogous to CLF, we used the model system DEBS1-TE, a bimodular PKS constructed by truncation of the erythromycin-producing PKS², which produces lactones 4 (major product) and 3 (minor product) (Fig. 3). A chimaeric PKS was created that contained the apparently acetate-specific loading module from the PKS for the polyether monensin (M. Oliynyk, unpublished data), fused to DEBS1-TE in the place of the normal loading module (Fig. 3). A mutant version was also produced in which the KSQ active-site glutamine (Gln 177) was specifically replaced by alanine. The chimaeric DEBS1-TE multi-enzyme and the Gln177Ala mutant were purified to homogeneity and the production of triketide lactones from acyl-CoA precursors was studied. Lactone 4 was produced by both enzymes whether or not a source of starter units was added, because of minor amounts of propionyl-CoA in the methylmalonyl-CoA used as the source of extender units²⁴. In the conventional view of the functioning of

a			b		
Fren	CLF	VVVDAGGLD	Nid	KSQ	VVDTGQSSSLV
Otc	CLF	ALVAEQAGGLD	Ty1	KSQ	VVDSAQSSSLV
Tcm	CLF	VFVTEAGGLD	Mon	KSQ	TVDTAQSSSLV
WhiE	CLF	VVAADAGGLD	Ery	KS1	SVDTAQSSSLV
Act	CLF	ALVAEQAGGLD			
Act	KS	MVSTGCTSGLD			

Figure 2 Active-site sequences of typical KS and CLF subunits from type II PKSs and KS and KSQ domains from type I PKSs. **a**, Alignment of CLF subunits of type II PKSs for frenolicin (Fren, GenBank accession no. L26338); oxytetracycline (Otc, Z25538); tetracenomycin (Tcm, M80674); *S. coelicolor* spore pigment (WhiE, X55942), and actinorhodin (Act, X63449); and of the actinorhodin KS (Act KS). Instead of the conserved cysteine in KS subunits, a glutamine or (rarely) a glutamate residue is found in CLF subunits (highlighted in black). **b**, Sequence alignment of KSQ domains from type I PKSs for niddamycin (Nid, AF016585), tylosin (Ty1, U78289), monensin (Mon; M. Oliynyk, unpublished data), and KS1 from the PKS for erythromycin (Ery, X62569). The position of the conserved cysteine in KS subunits is occupied by a glutamine in KSQ domains (highlighted in black).

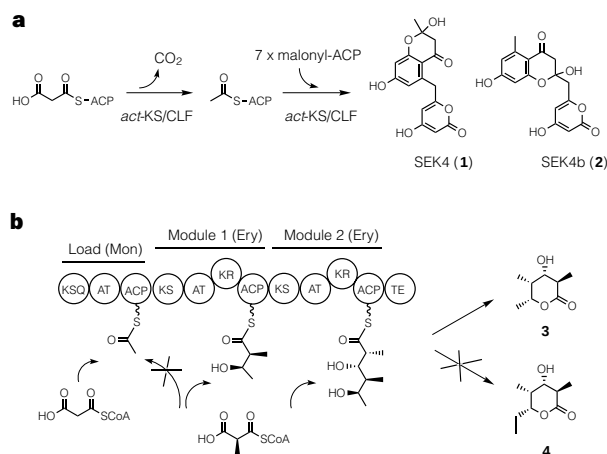


Figure 3 Functional analysis of KS/CLF and KSQ. **a**, The minimal *act* PKS system (malonyl-ACP, KS/CLF), which is capable of catalysing the formation octaketides 1 and 2, was used to study decarboxylase activity of mutant KS/CLF constructs *in vitro*. **b**, A chimaeric *mon*-loading module/DEBS1-TE construct was used for *in vitro* studies on the substrate specificity of the *mon* loading module. Incubation of purified protein with malonyl-CoA and (2*R*)-methylmalonyl-CoA gave rise to triketide lactone 3, which was not produced in the presence of acetyl-CoA and (2*R*)-methylmalonyl-CoA.

loading modules for type I PKSs, addition of acetyl-CoA would be expected to trigger specific polyketide synthesis, but it did not lead to the production of lactone 3 by either enzyme. In the presence of added malonyl-CoA, the unmutated enzyme produced lactone 3 at the same level as lactone 4; however, production of lactone 3 by the Gln177Ala mutant was reduced more than 10-fold compared with the unmutated enzyme. These results directly support the view that the glutamine residue is specifically important if not essential for polyketide biosynthesis, and that the KSQ domain functions in typical modular PKSs as a chain initiation factor with the same function as the CLF in aromatic PKSs, that is to say that the provision of starter units is through the specific decarboxylation of enzyme-bound extender units by the KSQ. Further work is required to establish whether the observed discrimination between acetate and propionate starter units in KSQ-containing loading modules is governed by the specificity of the AT domain of the loading module for either malonyl-CoA or methylmalonyl-CoA, or by the specificity of the KSQ domain, or both.

The discovery of the common role of CLF and KSQ as chain initiation factors provides an additional important mechanistic link between type I and type II PKSs, and between PKSs and FASs. Our results do not allow us to distinguish whether the active-site glutamine residue catalyses decarboxylation directly, or indirectly by its interaction with nearby amino-acid residues. If the effect proves to be direct, the glutamine residue might be acting as a general acid to polarize the thioester carbonyl group of the malonyl-ACP; alternatively, it could provide general base catalysis by direct bonding to the malonyl carboxyl group. Such roles would also be possible for a glutamate residue, which is found in place of glutamine in the CLF component of aromatic PKSs for spore pigments, typified by the *whiE* gene product (Fig. 2). Appreciation of the widespread importance of decarboxylation in the provision of starter units for polyketide assembly will probably be of major benefit for current efforts to engineer the biosynthesis of novel, potentially valuable polyketides. It may also assist in the analysis of

aromatic PKSs (such as those for frenolicin, oxytetracycline and daunomycin) which normally recruit non-standard starter units, but which, particularly in heterologous hosts, can accept acetate starter units³. It may be that the additional daunorubicin biosynthetic gene products DpsC and DpsD, and their frenolicin counterparts³, are needed to override (daunorubicin) or compete with (frenolicin) the supply of acetyl-ACP produced by CLF, but it remains to be determined whether these alternative starter units are also produced by acyl-ACP decarboxylation. □

Methods

Cloning and expression of chimaeric *mon-loading module DEBS1-TE constructs*

A chimaeric DEBS1-TE construct, containing the monensin PKS loading module (KSQ-AT-ACP domains; M. Oliynyk, unpublished data) fused to module 1 in DEBS1-TE, was obtained by a strategy previously described for attaching the avermectin PKS loading module (AT-ACP) to DEBS1-TE²⁵. The details of its construction, and that of the KSQ active-site mutant (Gln 177Ala) will be given elsewhere (P.F.L., J.C., J.S. and P.F.L., manuscript in preparation). The hybrid PKS genes were expressed in *Saccharopolyspora erythraea* JC2 using the expression plasmid pCJR24 (ref. 26). Purification of chimaeric DEBS1-TE multienzymes from *S. erythraea* JC2 and assays for synthesis of triketide lactones were essentially as described²⁴.

Cloning and expression of *act* PKS genes

Replacements of Cys 169 in *act* KS by Ala and Gln, respectively, and substitution of Ala for Gln 161 in *act* CLF were performed by site-directed mutagenesis (QuikChange, Stratagene) using a plasmid containing the linked *act* KS and CLF genes¹³ as the template for PCR. Expression of the coupled KS and CLF genes in *Streptomyces coelicolor* CH999 (ref. 4) and purification by nickel chelate affinity chromatography were as described¹³.

Assay for decarboxylation of malonyl-ACP

Conditions for reconstitution of the minimal *act* PKS have been reported¹³. After incubation of malonyl-ACP with purified KS/CLF, the acyl-ACP fraction was purified by reversed-phase HPLC and the acylation pattern of the ACP moiety was determined by ESMS as described²⁷.

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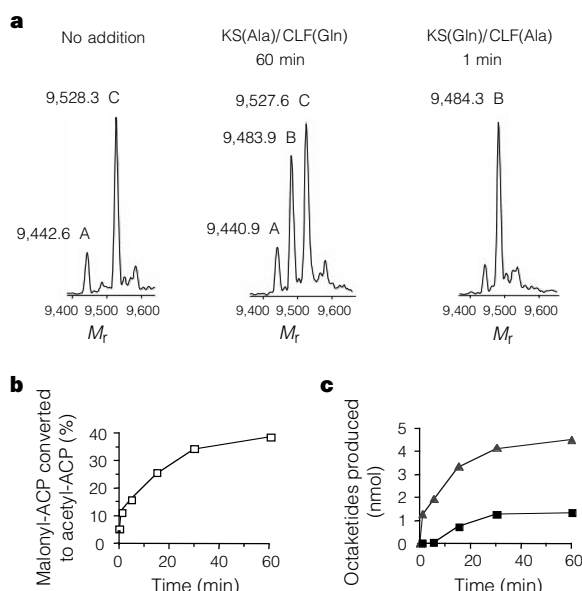


Figure 4 Conversion of malonyl-ACP to acetyl-ACP and polyketide production by KS/CLF mutants at 30 °C. **a**, ESMS transforms of (left) initial level of malonyl-ACP before decarboxylation and following incubation with either (middle) KS(Ala)/CLF(Gln) (60 min), or (right) KS(Gln)/CLF(Ala) (1 min). Expected values are A: 9441 *act*C17S *holo*-ACP; B: 9484 acetyl-ACP; C: 9528 malonyl-ACP. **b**, Time-course of decarboxylation of malonyl-ACP by KS(Ala)/CLF(Gln). **c**, Production of octaketides 1 and 2 by KS(Cys)/CLF(Ala), both in the presence (triangles) and absence (squares) of acetyl-ACP.

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Myosin VI is an actin-based motor that moves backwards

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Myosins and kinesins are molecular motors that hydrolyse ATP to track along actin filaments and microtubules, respectively. Although the kinesin family includes motors that move towards either the plus or minus ends of microtubules¹, all characterized myosin motors move towards the barbed (+) end of actin filaments². Crystal structures of myosin II (refs 3–6) have shown that small movements within the myosin motor core are transmitted through the 'converter domain' to a 'lever arm' consisting of a light-chain-binding helix and associated light chains^{5,6}. The lever arm further amplifies the motions of the converter domain into large directed movements^{3,5–7}. Here we report that myosin VI, an unconventional myosin^{8–12}, moves towards the pointed (–) end of actin. We visualized the myosin VI construct bound to actin using cryo-electron microscopy and image analysis, and found that an ADP-mediated conformational change in the domain distal to the motor, a structure likely to be the effective lever arm, is in the opposite direction to that observed for other myosins. Thus, it appears that myosin VI achieves reverse-direction movement by rotating its lever arm in the opposite direction to conventional myosin lever arm movement.

Although all myosin motors so far characterized move towards the barbed (+) end of actin filaments, there may well be cellular roles for oppositely directed myosins, and there are many unconventional myosins that have unknown functions and unusual primary structures^{2,8}. The simplest way to reverse the direction of movement would be to keep the basic motor with both its actin interface and core nearly identical to other myosins, but to attach the lever arm in such a way that the same movements of the motor core would rotate the lever arm in the opposite direction on actin, as compared with other myosins. We therefore compared the sequences of all known myosins, looking for large differences beginning in the converter domain and ending at the first light-chain-binding motif. Of the 15 known classes of myosins, the members of the myosin VI class were the only ones that fulfilled this criterion. They contain a 53-amino-acid insertion in the converter/lever arm region (Fig. 1); therefore, myosin VI was the best candidate for a reverse-direction myosin.

Class VI myosins were first identified in *Drosophila melanogaster*⁹ and were subsequently found to be expressed in species from *Caenorhabditis elegans* to human^{8,10–12}. Myosin VI has a single

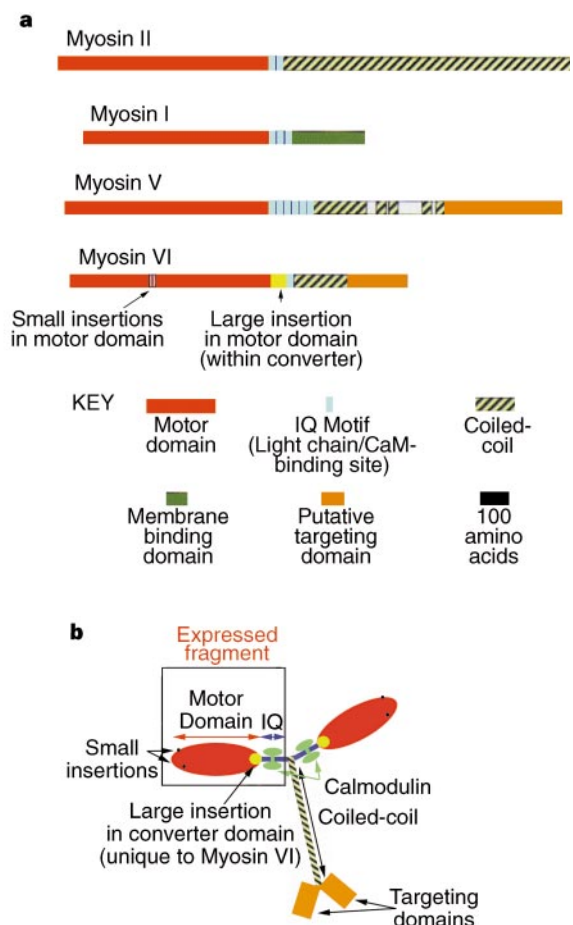


Figure 1 Myosin VI. **a**, Comparison of the primary structures of four classes of myosins. Two small insertions and one large insertion at the end of the motor domain (within the converter) comprise the main differences between the motor domain of myosin VI and those of other members of the myosin family. Conventional myosin (myosin II from smooth muscle) and two unconventional myosins (brush border myosin I and myosin V) all move toward the barbed (+) ends of actin filaments. There are differing numbers of light-chain/CaM-binding sites (IQ motifs) among these classes, as well as highly divergent C-terminal regions (to the right of the IQ motifs). **b**, The native myosin VI molecule, with a box around the truncation used in this study. The expressed fragment had additional amino acids after the IQ motif (C terminus) for purification and motility measurements (see Methods).

calmodulin (CaM)-binding site following the motor domain and contains a coiled-coil region as well as globular carboxy-terminal tail^{2,8,10}. Myosin VI is found in a variety of cell types^{13–15}. The myosin VI gene (*myo6*) is defective in the deaf mouse, Snell's waltzer¹¹, indicating that myosin VI may be required for the function of sensory hair cells^{11,13}. Immunolocalization studies suggest that, in a variety of cell types, myosin VI is involved in vesicular transport^{13,15}.

We co-expressed CaM with a single-headed pig myosin VI construct coding for the region including the motor domain and the helical CaM-binding domain ('IQ' motif⁸) in insect SF9 cells (Fig. 1b). The purified protein had a single CaM bound per heavy chain. At 25 °C, the expressed myosin displayed maximal actin-

activated ATPase activity of 0.8 per head s^{-1} ($K_{ATPase} = 3 \mu M$), and moved actin filaments at an average velocity of $58 \pm 10 \text{ nm s}^{-1}$.

To determine definitively the direction of movement, we modified the standard *in vitro* motility assay¹⁶ to create actin filaments with labelled pointed ends by polymerizing off the barbed end of rhodamine-labelled, cross-linked F-actin nuclei. We then stabilized the filaments with fluorescein isothiocyanate (FITC)-phalloidin. The resulting actin filaments were green throughout, with a red cap at the pointed end. These filaments confirmed that myosin V, like myosin I and myosin II, moves towards the barbed (+) end of actin filaments¹⁷. As shown in Fig. 2a, a surface-bound, truncated myosin V moved actin filaments so that the pointed end was at the front of the moving filament (that is, the myosin moved away from the pointed end, towards the barbed end). When filaments from the same preparation were put on myosin VI-coated surfaces, they moved in the opposite direction, showing that myosin VI moves toward the pointed (–) ends of the filaments (Fig. 2b). Data for a number of polarity-marked filaments are summarized in Fig. 2c), with positive velocity indicating movement toward the barbed (+) end and negative velocity indicating movement toward the pointed (–) end of an actin filament. All data confirm that myosin VI moves in the opposite direction to that of previously studied myosins.

We decorated actin filaments with our myosin VI construct for analysis by cryo-electron microscopy and image reconstruction to gain structural insight into the reverse-direction motility of myosin VI. We calculated three-dimensional maps of the truncated myosin VI bound to actin filaments in the presence and absence of ADP (Fig. 3a, b); these reveal that this myosin has an unusual shape.

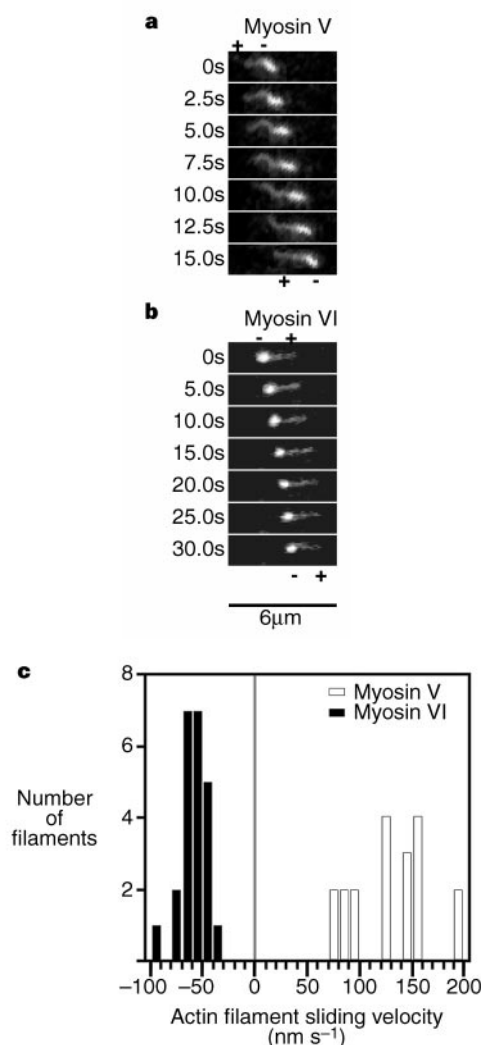


Figure 2 Determination of direction of myosin movement. Panels display the same spatial field at the relative times shown on the left. Within each panel is a single actin filament (visualized using FITC-phalloidin) moving in a conventional *in vitro* motility assay¹⁷. The bright (rhodamine/FITC-labelled) tip on each filament marks the pointed (–) end of the filament. **a**, Movement of actin by a myosin V construct containing the motor domain and one CaM/light-chain-binding site. The actin filament moves with the pointed (–) end leading, indicating that the myosin V moves towards the barbed (+) end. The filament sliding velocity was 140 nm s^{-1} (25 °C). **b**, Movement of actin by a myosin VI construct containing the motor domain and its one CaM-binding site. The actin filament moves with the pointed (–) end trailing, indicating that the myosin VI moves toward the pointed (–) end. The filament sliding velocity was 60 nm s^{-1} (25 °C). **c**, Histogram of the velocities of polarity-marked actin filaments moving on either myosin V or myosin VI. Movement of myosin towards the barbed (+) end was arbitrarily defined as positive velocity and movement toward the pointed (–) end as negative velocity.

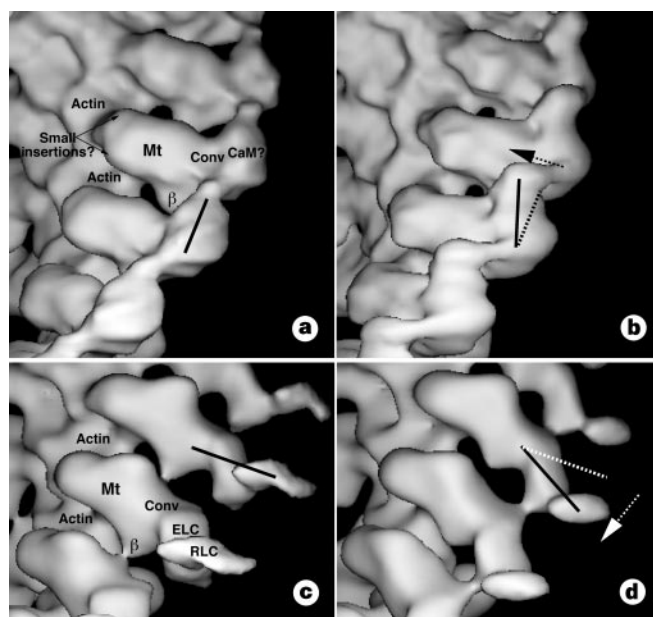


Figure 3 Comparison of ADP (left) and rigor (right) 3D maps of myosin VI (**a**, **b**), and smooth muscle myosin II (**c**, **d**) attached to F-actin. All maps are at the same scale; the vertical extent of the maps in **a** and **b** is $\sim 250 \text{ Å}$. Solid lines indicate the long axes of the CaM- or light-chain-binding domains of the two myosins. Arrows show the direction of rotation of the domains in the transition between the ADP and rigor states. The data in **c** and **d** is taken from ref. 7. The rotation was roughly $15\text{--}20^\circ$ for myosin VI, and $20\text{--}25^\circ$ for myosin II. β denotes N-terminal β -barrel domains; Mt overlays the approximate positions of the cores of the myosin motors. Small Insertions? highlights regions of density found only in myosin VI which are the possible locations of the 9-amino-acid (lower arrow) and 13-amino-acid (upper arrow) inserts between the motor core and actin interface. Conv denotes the approximate locations of the converter domains. CaM? denotes the probable CaM density within myosin VI. ELC denotes the essential light chain and RLC the regulatory light chain of myosin II, which together constitute the effective lever arm.

Fig. 3a shows our single-headed myosin VI in the presence of 5 mM Mg-ADP. The myosin VI motor domain has the same geometry of attachment to F-actin as conventional myosin (Fig. 3c), but it has a number of differences that most probably reflect small differences in the primary structure. For example, myosin VI contains two small insertions of roughly 9 and 13 amino acids in a region between the nucleotide pocket and the actin-binding interface (insertions beginning at positions corresponding to residue 317 or 343 of the chicken skeletal myosin II sequence; see Figs 1 and 3a). The insertion of 53 amino acids, which is nominally between the converter domain and light-chain-binding helix, may in fact be a component of a radically divergent myosin VI converter domain. Indeed, the most notable differences between this map and maps of other myosins (myosin I, myosin II or myosin V) are in the light-chain-binding region, which has an unusual shape and orientation. At the resolution of this study, the location of the additional 53-amino-acid insert is unclear. Likewise, the CaM light chain is not unambiguously resolved, but we tentatively assign CaM to the region with an elongated, asymmetric shape (Fig. 3a).

We determined the unusual functional aspect of the structure by comparing nucleotide-bound (ADP) and nucleotide-free (rigor) states of the motor. For some isoforms of either myosin II (ref. 7) or myosin I (ref. 18), a part of the myosin conformational change on actin is associated with ADP dissociation. Thus, a comparison of the ADP and rigor structures of our myosin VI isoform on actin could show this movement, together with the structural features that move and the direction of the movement. Unlike other myosins in which the effective lever arm (light-chain-binding domain) projects out at a right angle to the filament axis (for example, brush border myosin I (ref. 18)) or towards the barbed end of the filament (for example, myosin II (ref. 7); Fig. 3c, d), the myosin VI lever arm (the putative CaM-binding domain) appears to project towards the pointed end of the actin filament (Fig. 3a, b). Furthermore, comparing the maps of ADP and rigor structures (Fig. 3a, b), shows that the lever arm clearly rotates in the opposite direction to myosin II when ADP is released from the head (Fig. 3c, d). Although little of the rotation associated with ADP release appears to be parallel to the actin filament, we hypothesize that the ADP-induced rotation observed is simply the final small fraction of the overall rotation associated with the myosin VI powerstroke; as is the case with smooth muscle myosin II (refs 5, 7).

Achieving reversal of myosin direction on actin by altering the converter domain/lever arm is analogous to the recently proposed strategy underlying direction determination within the kinesin motor family^{19–21}. Directionality within the kinesin family is primarily a function of the neck region of the kinesin/ncd motors, with possible contributions from the stalk and, to a lesser extent, the motor core²¹. The neck and stalk elements must be involved in the different positioning of the second head of kinesin compared to ncd that is observed in cryo-electron microscopy reconstructions^{20–23}. Although it is not clear that either of these kinesin structural elements functions as a true lever arm, the kinesin neck may be functionally analogous to the converter domain of myosin. Thus, the myosins and kinesins appear to use a similar general strategy to achieve reversal of movement. In each family, the motor core is preserved and direction of movement depends on how the same nucleotide-mediated movements are subsequently interpreted and amplified by other regions of the molecule.

Myosin VI has evolved to provide reverse-direction movement (that is, towards the pointed (–) end) on an actin filament. Sequence alignments indicate that it may be the only myosin class with a strategy of altering the converter domain, and thus may be unique among myosins in its direction of movement. This motor represents a means of providing (in conjunction with other classes of myosins) bidirectional movement on polarized actin filaments or filament bundles. A full understanding of the cellular importance of this motor awaits further investigation. □

Methods

Protein engineering and preparation

Myosin VI was expressed using the baculovirus/SF9 cell expression system. The expression and purification were as described²⁴. To create the recombinant virus used for expression, we constructed a single-headed myosin VI by truncating the complementary DNA coding for pig myosin VI (ref. 10) after the codon corresponding to Gly 840. This construct encompassed the motor domain and the one CaM-binding site of myosin VI. Either a 'Flag' tag DNA sequence (encoding GDYKDDDDK)²⁵ or sequential 'myc' (encoding EQKLISEEDL)²⁶ and Flag tags were appended to the truncated myosin VI coding sequence. A stop codon followed the Flag tag in both cases. We used the Flag tag for purification, and the Myc tag in *in vitro* motility assays to couple the myosin to nitrocellulose surfaces using an antibody against the Myc epitope. A full-length cDNA for chicken CaM was co-expressed with the truncated myosin VI heavy chain. The recombinant, truncated chicken myosin V (ref. 27) used in the motility experiments summarized in Fig. 2 was produced in the same manner. It was truncated after its first CaM/light-chain-binding site, at Arg 792. Both Myc and Flag tags were appended for motility assays and purification, respectively.

Functional assays

Determination of actin-activated ATPase activity (at 25 °C in 20 mM KCl) followed published procedures^{24,28}, as did the determinations (at 25 °C in 50 mM KCl) of actin-filament sliding velocity^{16,24}. To use the *in vitro* motility assay to determine direction, actin filaments were generated that had red (rhodamine) labels at their pointed (–) ends. This involved the preparation of crosslinked, fluorescent actin nuclei by first dialysing 125 µM F-actin into 0.1 M KCl, 2.5 mM Na₂B₄O₇, 1 mM MgCl₂, 0.2 mM ATP, pH 9.0. Next, 1 mol *p*-phenylenedimaleimide (as a 10 mM solution in dimethylformamide (DMF)) was added per mol actin monomer, and crosslinking proceeded for 1 h at 25 °C (ref. 29). A fourfold excess of tetramethylrhodamine isothiocyanate was added (as a 33 mM solution in DMF), and the labelling reaction carried out for 2 h in the dark at 4 °C. The material was dialysed in the dark for 16 h against 5 mM Tris-HCl, 0.2 mM ATP, 0.2 mM CaCl₂, 0.2 mM NaN₃, pH 7.5, and then centrifuged for 30 min at 200,000 g, yielding a soluble supernatant and a depolymerization-resistant pellet of fluorescent F-actin. After the supernatant was polymerized by salt, pelleted, and redialysed against depolymerizing buffer, we obtained a second depolymerization-resistant pellet. The depolymerization-resistant pellets, which were highly enriched in actin dimers and higher oligomers as shown by SDS-PAGE, were used for rhodamine-labelled actin nuclei. The resuspended pellets were sheared through a 25-gauge needle to produce short filaments, and precipitated free dye was removed by centrifugation at 16,000g for 15 min. We added unlabelled actin to these nuclei at two different concentrations below the critical concentration for polymerization from the pointed end, but above the critical concentration for the barbed end (0.10 or 0.25 µM). Polymerization took place overnight. Filaments were stabilized by the addition of FITC-phalloidin, and inspection confirmed that a small percentage of the resulting FITC-labelled actin filaments contained a rhodamine tip (breakage of filaments probably creates many unlabelled nuclei for polymerization). The tip was visible in the rhodamine channel of a fluorescence microscope equipped with an intensified CCD camera. The entire filament was visible in the FITC channel. With dual excitation of rhodamine and FITC, the tip was brighter than the rest of the filament (Fig. 2), as it contained multiple rhodamine labels per actin monomer as well as FITC-phalloidin.

Electron microscopy and image analysis

Recombinant myosin VI at 2–5 mg ml^{–1} in 10 mM imidazole, pH 7.0, 50 mM KCl, 2 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT was used to decorate platelet actin filaments adsorbed to holey carbon support films on electron microscopy grids. For the nucleotide-free condition, apyrase was added to the solution (final concentration ~10 U ml^{–1}) on the grid just before blotting and freezing in liquid ethane slush. For the ADP condition, the myosin buffer was supplemented with 5 mM Mg-ADP. We used a Gatan cold stage, operating at –180 °C, to examine the grids in a Philips CM200 FEG electron microscope. Images of filaments spanning holes in the support film were recorded at 40,000× at 1.1–2.0 µm under focus. Selected images of well ordered filaments were digitized with spot and step sizes equivalent at 4.97 Å at the specimen. Image analysis, averaging and calculation of 3D maps were carried out essentially as described³⁰. Data from 43 (8,586 subunits) and 27 filaments (4,617 subunits) were averaged for the ADP and rigor structures, respectively. The resolution of the two final maps is 20–25 Å as judged from phase agreement along the layer lines. A full description of the data will be published elsewhere. The 3D maps were visualized with Volvis.

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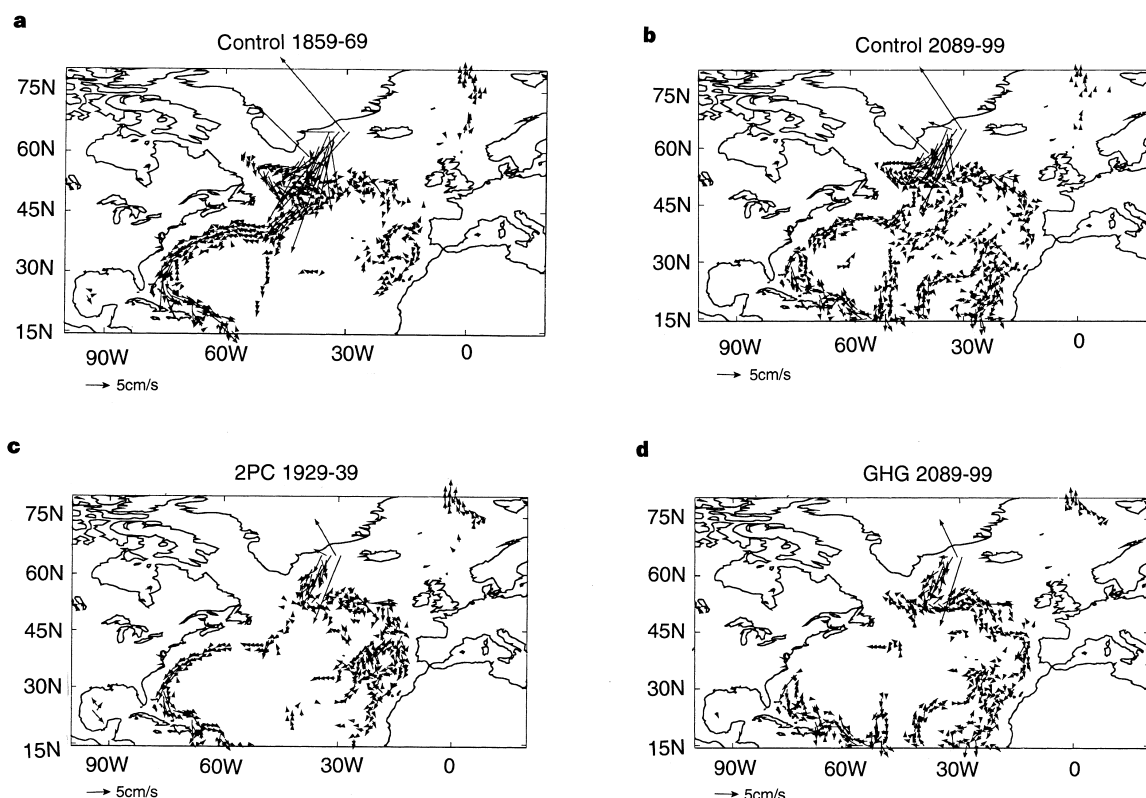
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Richard A. Wood, Ann B. Keen, John F. B. Mitchell
& Jonathan M. Gregory

Nature **399**, 572–575 (1999)

The scale vectors shown at the bottom left of each panel in Fig. 2 were the wrong size as published; the corrected figure is shown below. □



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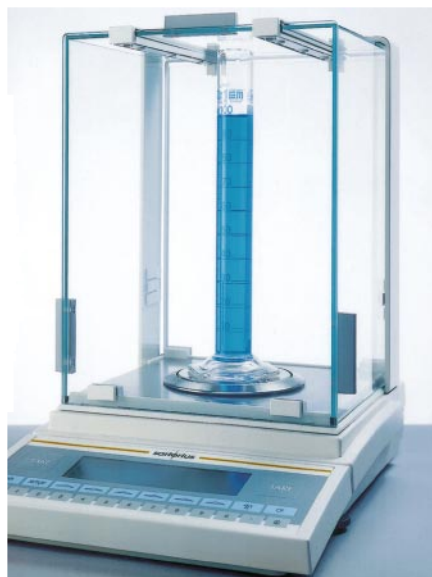
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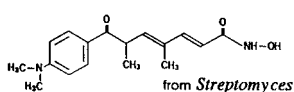
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